UNIVERSIDADE DE LISBOA

FACULDADE DE MOTRICIDADE HUMANA

BODY COMPOSITION IN

NON-ALCOHOLIC FATTY LIVER DISEASE PATIENTS

USEFULNESS OF BODY COMPOSITION CLINICAL MARKERS IN THE ASSESSMENT OF BODY FAT CONTENT AND DISTRIBUTION AND RELATED AUTONOMIC CARDIAC CONTROL

Dissertação com vista à obtenção do grau de Doutor em

Motricidade Humana na especialidade de Atividade Física e Saúde

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2014

Agradecimentos (acknowledgements)

Porque nenhum trabalho desta dimensão pode ser realizado sem a ajuda e o apoio de outras pessoas e instituições, expresso aqui o meu profundo agradecimento a todas elas. Em primeiro lugar os meus agradecimentos vão para a minha orientadora, a Professora Doutora ***Helena Santa-Clara****, pelo seu apoio, amizade, confiança, estímulo, pela forma como acolhe os seus doutorandos e luta por eles, pela forma como resolve problemas e como transforma questões complicadas em soluções simples, por ser uma das pessoas mais verdadeiras que conheci, mais fiel a si própria e que eu mais respeito (ganho e merecido). Em segundo lugar agradeço à minha co-orientadora, a Professora Doutora* ***Helena Cortez-Pinto*** *(gastroenterologista no Hospital de Santa Maria e Faculdade de Medicina – Universidade de Lisboa), pelo seu apoio (insubstituível), pela forma como me recebeu (sem me conhecer de lado nenhum), pela confiança que depositou em mim e por todo o seu esforço para que este trabalho fosse bem-sucedido, com a qualidade desejada. Agradeço ainda aos elementos do* ***Júri*** *pela sua disponibilidade.*

Agradeço à instituição ***Escola Superior de Desporto de Rio Maior*** *do Instituto Politécnico de Santarém (onde desenvolvo a minha atividade profissional desde 2000), em particular aos três diretores que a ESDRM já teve desde a sua génese: Professores Doutores* ***José Rodrigues****,* ***Abel Santos*** *e* ***Rita Santos Rocha****, pelo apoio institucional e ajuda financeira a este projeto de doutoramento. Agradeço especialmente à minha atual diretora, estimada colega e amiga pessoal Rita Santos Rocha pela sua confiança continuada no meu trabalho, pelo seu estímulo ao longo do tempo, pela preciosa colaboração na revisão final do presente documento, e sobretudo pela amizade. Agradeço à instituição* ***Faculdade de Motricidade Humana*** *da Universidade de Lisboa pelo acolhimento enquanto estudante de mestrado (2002-2006) e de doutoramento (2008-2014).*

Um agradecimento especial também às pessoas que estiveram diretamente envolvidas neste projeto: ao Professor Doutor ***Luís Bettencourt Sardinha*** *pelo seu conhecimento, experiência e rigor e pelo tempo que dedicou para ajudar a elevar sempre o nível do meu trabalho, ao Professor Doutor* **Bo Fernhall** (Dean of the College of Applied Health Sciences – University of Illinois) *pelo seu apoio, confiança e rigor científico, ao Dr.* ***José Silva-Nunes*** *(endocrinologista do Hospital Curry Cabral e doutorando solidário com o presente projeto) pela sua confiança no projeto e pela sua ajuda no recrutamento da amostra, aos Dr.* ***Pedro Sousa*** *e Drª* ***Rita Calé*** *(à data internos de cardiologia no*

***Agradecimentos* (acknowledgements)**

Hospital de Santa Cruz) pela sua paixão à sua profissão, pelo seu voluntarismo e pela sua inestimável colaboração nas provas de esforço, à Professora Doutora ***Cristina Bento*** *e a todos os colegas que colaboraram nas colheitas e acomodação de amostras de sangue e saliva (parâmetros recolhidos no âmbito do presente projeto mas não tratados nesta tese), e aos colegas de doutoramento Mestre* ***Xavier Melo****, à Professora Doutora* ***Maria da Lapa Rosado****, à Professora Doutora* ***Diana Santos****, à Mestre* ***Catarina Matias****, às Professoras Doutoras* ***Teresa Tomás*** *e* ***Margarida Carrolo*** *pela colaboração continuada ao longo do duríssimo processo de recolha de dados e tratamento dos mesmos. Agradeço igualmente a todos os que voluntariamente participaram na* ***amostra*** *deste estudo. Um agradecimento ainda aos professores e amigos que, em momentos particulares, terão sido especialmente importantes para a consecução deste trabalho, à Professora Doutora* ***Isabel Fragoso****, à Professora Doutora* ***Analiza Silva****, ao Professor Doutor* ***Francisco Alves****, à Professora Doutora* ***Filomena Carnide****, à Professora Doutora* **Tracy Baynard** (Assistant Professor in College of Medicine – University of Ilinois*); à Professora Doutora* ***Fátima Batista****; ao Professor Doutor* ***Pedro Teixeira;*** *à Mestre* ***Helo Isa André****, e à Professora Doutora* ***Teresa Branco***.

Agradeço a todos os ***Amigos e Colegas*** *da Escola Superior de Desporto de Rio Maior, que me apoiaram em vários momentos, em particular aos Professores Doutores* ***João Moutão****,* ***Susana Franco****,* ***Rita Santos Rocha****,* ***Susana Alves****,* ***Vera Simões****,* ***Félix Romero****,* ***David Catela****,* ***Teresa Bento****,* ***Luís Cid*** *e aos mestres* ***Fátima Ramalho****,* ***Marco Branco****,* ***Xavier Melo*** *e* ***Ana Paula Seabra****.*

Não posso deixar de agradecer às duas instituições onde redigi a quase totalidade do presente documento, aos seus funcionários e às respetivas câmaras municipais que as governam: a ***Biblioteca Municipal Loureano Santos*** *e respetiva Câmara Municipal de Rio Maior, e a* ***Biblioteca Municipal de Castelo Branco*** *e respetiva Câmara Municipal de Castelo Branco, um "bem hajam" pelo acolhimento e pelas excecionais condições de trabalho proporcionadas.*

Por último, mas sempre em primeiro lugar, agradeço à minha Família - à minha mulher ***Sílvia****, aos meus pais* ***Constança*** *e* ***Joaquim****, aos meus sogros* ***Amália*** *e* ***Luís****, aos meus Irmãos* ***Pedro*** *e* ***João****. Aos meus filhos* ***Manuel*** *e* ***Maria*** *(M&Ms) pelo desafio constante e pelo amor incondicional, e pela compreensão pela ausência nos momentos dedicados ao estudo e elaboração desta tese.*

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Thesis abstract

The present thesis includes five original studies focusing on body composition (BC) in non-alcoholic fatty liver disease (NAFLD) patients, mainly in a clinical perspective, directed to clinical practice. The first study of this thesis aimed at analyzing the relation between body fat (BF) content and distribution, as assessed by dual energy x-ray absorptiometry (DXA), and cardiac autonomic control, more specifically with heart rate recovery after a maximal exercise test, which is an indirect clinical marker of parasympathetic reactivation, also known to be a strong risk factor for overall and cardiac mortality. The second study focused on the utility of waist circumference (WC) measurement, as a predictor of both BF content and distribution, and also on the comparison of different WC measurement protocols based on biological criteria, protocols' precision and practical criteria, aiming to identify a preferential measurement protocol to be used in NAFLD patients. The third and fourth studies focused on the influence of using different WC measurement protocols in the relation of both waist-to-hip ratio (WHR) and waist-to-height ratio (WHtR) with BF content and distribution, as assessed by DXA, and aimed to identity a preferable measurement protocol. The fifth study focused on body indexes and circumferences usefulness as surrogates of BF content and distribution and aimed at identifying if there is a preferential clinical predictor of both BF content and distribution, as compared to the commonly used body mass index, in NAFLD patients. The results obtained confirmed the strong relation between BC and cardiac autonomic control and showed that BF distribution is more important than BF content in explaining cardiac autonomic control variation. It was also possible to conclude that WC measured just above the iliac crest seem preferable to be used in NAFLD patients, either singly or included in body indexes such as WHR or WHtR, mostly due to practical criteria but also because of it strong correlation with both BF content and distribution. WHtR appears to be the best BF content and distribution surrogate to be used in clinical practice with NAFLD patients. WC alone is a good practical alternative, when simplicity and time saving are important instrument/method selection criteria.

Resumo da tese

A presente tese integra cinco investigações originais que se centram no estudo da composição corporal (CC) em pacientes com doença do fígado gordo não-alcoólico (DFGNA), numa perspetiva eminentemente clínica e direcionada para a prática. Um primeiro estudo visou analisar a relação da quantidade e distribuição da massa gorda corporal (MG), avaliada por densitometria por raio-X de dupla energia (DXA), com o controlo autonómico cardíaco, mais especificamente com um indicador indireto da reativação do sistema nervoso parassimpático, que é a frequência cardíaca de recuperação após um esforço máximo, que também é um forte fator de risco para mortalidade. O segundo estudo visou avaliar a utilidade da medição do perímetro da cintura, isoladamente, como preditor da quantidade e distribuição de MG, em pacientes com DFGNA, e comparar os resultados e os procedimentos da medição do perímetro da cintura realizada segundo diferentes protocolos de medição de modo a identificar um protocolo preferencial. O terceiro e quarto estudo pretenderam avaliar o impacto da utilização do perímetro da cintura obtido segundo diferentes protocolos de medição na performance da razão cintura/anca e da razão cintura/altura, enquanto indicadores clínicos, duplamente indiretos, de quantidade e distribuição de MG. O quinto e último estudo deste trabalho teve como objetivo avaliar a relação de perímetros e índices corporais com a quantidade e distribuição de MG, em pacientes com DFGNA, e procurou identificar a existência de alternativas preferenciais à utilização do índice de massa corporal. Os resultados encontrados no presente trabalho permitem confirmar que a CC está fortemente relacionada com o controlo autonómico cardíaco, em pacientes com DFGNA, e que, nessa relação, a distribuição de MG parece ser mais determinante do que a sua quantidade absoluta e relativa. Também foi possível concluir que o perímetro da cintura medido imediatamente acima da crista ilíaca parece ser a melhor metodologia para ser utilizada com esta população, sobretudo por razões de ordem prática, mas também pelo seu desempenho na relação com quantidade e distribuição de MG, quer quando utilizado isoladamente como quando integrado em índices corporais, como a razão cintura/ anca ou a razão cintura/ altura. A razão cintura/altura parece ser a melhor alternativa para ser usada como preditor da quantidade e distribuição de MG em pacientes DFGNA, sendo que o perímetro da cintura também é uma boa alternativa sobretudo por razões de ordem prática.

List of Abbreviations

Abd - Abdominal;

ANS - Autonomic nervous system;

Append - Appendicular;

Arm-C - Arm circumference;

BAI - Body adiposity index;

BF - Body fat;

BC - Body composition;

BMI - Body mass index;

CAbd - Central abdominal;

CAD - Coronary artery disease;

Calf-C - Calf circumference;

CAN - Central autonomic network;

CDC - Centers for disease control and prevention;

CHD - Coronary heart disease;

CIPER - Interdisciplinary Center for the Study of Human Performance;

COV - Coefficient of variation;

CRP - C-reactive protein;

CSEP - Canadian Society of Exercise Physiology;

CT - Computed tomography;

CV - Cardiovascular;

CVD - Cardiovascular disease;

DXA - Dual-energy X-ray absorptiometry;

DM - Diabetes mellitus;

ECG - Electrocardiogram;

FFM - Fat free mass;

GLUT 4 - Glucose transporter type 4;

GXT - Graded exercise test;

Hip-C - Hip circumference;

HSL - Hormone sensitive lipase;

HR - Heart rate;

HR1 - Heart rate at 1 minute after maximal effort;

HR2 - Heart rate at 2 minute after maximal effort;

HRmax - Maximal heart rate;

HRR - Heart rate recovery;

HRR1 - Heart rate recovery at 1 minute after maximal effort;

HRR2 - Heart rate recovery at 2 minute after maximal effort;

IL-6 - Interleukin 6;

IR - Insulin resistance;

ISAK - International Society for the Advancement of Kinanthropometry;

L4 - Fourth lumbar vertebra;

LabES - Exercise and health laboratory;

LPL - lipoprotein lipase;

NAFLD - Non-alcoholic fatty liver disease;

NASH - Non-alcoholic steatohepatitis;

NEFA - Non esterified fatty acids;

NHANES - National Health and Nutrition Examination Survey;

NIH - National Institutes of Health;

LIST OF ABREVIATIONS

MONA LISA - Most obesities known are low in sympathetic activity;

MRI - Magnetic resonance imaging;

MRS - Magnetic resonance spectroscopy;

PNS - Parasympathetic nervous system;

ROI - Region of interest;

ROS - Reactive oxygen species;

SNS - Sympathetic nervous system;

T2DM - Type 2 diabetes mellitus;

Thigh-C - Thigh circumference;

TNF-α - Tumor necrosis factor alfa;

VLDL - Very low density lipoprotein;

WC - Waist circumference;

WC1 - Waist circumference measured at the narrowest torso;

WC2 - Waist circumference measured just above iliac crest;

WC3 - Waist circumference measured at the mid distance between iliac crest and lowest rib;

WC4 - Waist circumference measured at the navel;

WCmp - Waist circumference measurement protocol;

WHO - World Health Organization;

WHR - Waist-to-hip ratio;

WHR1 - Waist-to-hip ratio using WC1;

WHR2 - Waist-to-hip ratio using WC2;

WHR3 - Waist-to-hip ratio using WC3;

WHR4 - Waist-to-hip ratio using WC4;

WHtR - Waist-to-height ratio;

WHtR1 - Waist-to-height ratio using WC1;

WHtR2 - Waist-to-height ratio using WC2;

WHtR3 - Waist-to-height ratio using WC3;

WHtR4 - Waist-to-height ratio using WC4;

LIST OF ABREVIATIONS

Chapter 1 – Introduction

"Presentation of this thesis, including its main focus and purposes as well as its organization"

With the present thesis entitled "Body Composition in Non-Alcoholic Fatty Liver Disease Patients - Usefulness of Body composition and Fat Distribution Markers and their Relation with Autonomic Nervous System Cardiac Regulation" (*Composição Corporal na Doença do Fígado Gordo Não-alcoólico – Utilidade de Indicadores de Composição e Distribuição de Gordura corporal e a sua Relação com a Regulação Cardíaca pelo Sistema Nervoso Autónomo*), the author aims to obtain the Doctoral Degree in Human Kinetics in the specialty of Physical Activity and Health (*Doutoramento no ramo de Motricidade Humana na especialidade de Atividade Física e Saúde*) by the Faculty of Human Kinetics (*Faculdade de Motricidade Humana*) – University of Lisbon (*Universidade de Lisboa*), Portugal. The project for the present thesis was submitted to the *Fundação para a Ciência e Tecnologia – Ministério da Educação e Ciência,* for an individual doctoral scholarship, which was awarded (SFRH / BD / 41173 / 2007). The study was developed and conducted at the Interdisciplinary Center for the Study of Human Performance (CIPER), at the Faculty of Human Kinetics - University of Lisbon, Portugal.

This work results from the will and effort to contribute to enlighten selected features of clinical routine assessments of body composition (BC) of patients diagnosed with Non-Alcoholic Fatty Liver Disease (NAFLD). In order to fulfill this purpose, a mix of laboratory and field methods was used for the assessment of BC and autonomic nervous system status. The relation of different body fat (BF) depots and ratios with a selected mortality and cardiovascular risk related autonomic nervous system marker was assessed, and therefore one perspective of the relevance of BC assessment in these patients was discussed. The usefulness of clinical methods and specific markers and indexes for the assessment of BC to use in daily clinical practice with the studied patients was then evaluated and discussed. Ultimately, suggestions were made concerning the inclusion of selected methods for efficient assessment of the BC in NAFLD patients in clinical settings.

1.1. Research rationale

NAFLD is an increasingly recognized condition that may progress to end-stage liver disease. The prevalence of NAFLD is estimated to range up to over 30% of general population in western countries tough accurate epidemiologic data are still not available in many countries (1-5). If we consider only the obese population, particularly in abdominal or in morbidly obesity, the prevalence raises significantly ranging up to 100% (1-3). Because of its' strong association with obesity, mainly central obesity, and with impaired glucose metabolism, it is expected that NAFLD will increase strongly, establishing a parallel with the pandemic dimension of the mentioned metabolic disorders

(5, 6). NAFLD is also considered to be the hepatic manifestation of the metabolic syndrome (7), and it is known that NAFLD patients are at greater cardiovascular risk when compared with the general population (8).

BC, particularly specific BF distribution adverse phenotypes (central, abdominal or visceral obesity), may be paramount in the aetiology of metabolic disorders such as NAFLD (9), and is known to be associated to increased overall and cardiovascular mortality as well as mortality and cardiovascular risk factors (5, 10-17). Obesity seems to be also somewhat related to important mortality and cardiovascular risk related autonomic nervous system (ANS) markers (18). As a part of the human nervous system that has been shown to be responsible for many important metabolic body functions, including adipocyte, heart and liver functions (19, 20), the ANS may be an important piece of the puzzle in the understanding of metabolic disorders and may also be an important target of intervention. Data on the relation of BC and heart rate recovery (HRR), a selected marker of ANS functioning, is scarce, particularly in subpopulations such as NAFLD patients where it is mostly absent. Although being utmost important in the study of metabolic disorders, the study of BC is not much explored in the mentioned subpopulation, plus specific and comprehensive guidelines for the assessment of BC in NAFLD patients in the clinical setting are lacking. Most often the only BC marker studied is the body mass index (BMI) which, although easy to measure and proven useful in epidemiological approaches, has known limitations (21, 22). One important BC surrogate that arises as a good complement, and possibly an alternative, in BC assessment in clinical settings is the waist circumference (WC) (17, 23). However it has been difficult to standardize measurement procedures, particularly concerning the anatomical landmarks for the measurement. Other BC clinical markers are gaining importance but again, standardization may be a strong impediment for broad implementation. The recently suggested body adiposity index seems less exposed to the mentioned standardizations difficulties (24) however it has not yet proven significant predictive superiority as compared to existing surrogates (25). These limitations may be restraining the implementation of additional or alternative measures in clinical BC appraisals. Any contribution to enlighten the mentioned miasmas in the current knowledge may be valuable contribution for the clinical management and possible prognostic of epidemic metabolic disorders such as NAFLD. Studies focusing on these miasmas are needed before recommendations and guidelines can be properly formulated.

1.2. Research questions

Literature focusing on the study of human BC is abundant but studies focusing on the study of BC in NAFLD patients are not so. In order to contribute for the understanding of the importance of BC in NAFLD patients and to increase the information available to support comprehensive guidelines for BC assessment of NAFLD patients in the clinical setting, we sought to formulate the following questions:

1 - Is BF content and distribution associated with ANS in NAFLD patients?

2 - What is the best WC measurement protocol to be used in clinical practice as a surrogate of BF content and distribution in NAFLD patients?

3 - What is the best WC measurement protocol to be used in clinical practice to calculate waist-to-high ratio as a surrogate whole and regional BF in NAFLD patients?

4 - What is the best WC measurement protocol to be used in clinical practice to calculate waist-to-hip ratio as a surrogate of BF content and distribution in NAFLD patients'?

5 - What are the best body index and/or circumference to be used in clinical practice as a surrogate of BF content and distribution in NAFLD patients?

1.3. Purposes of this research

The main purposes of this thesis were to contribute for the understanding of the importance of BC in NAFLD patients as well as to increase the information available for building comprehensive guidelines for BC assessment of NAFLD patients in the clinical setting. With this in mind we formulated the following specific purposes of the present study:

1- To determine if, and to what extent, specific markers of BC and BF distribution, are related with a selected mortality and cardiovascular risk related ANS marker in NAFLD patients.
2- To find which of the most used WC measurement protocol is preferable to be used in clinical practice with NAFLD patients.

3- To analyze whether the most used WC measurement protocol affect the strength of association between waist-to-height ratio and both, whole and central BF in NAFLD patients.
4- To analyze whether the most used WC measurement protocol affect the strength of association between waist-to-hip ratio and BF content and distribution in NAFLD patients.
5- To analyze how body circumferences and indexes perform as surrogate of whole and regional BF content and BF distribution in NAFLD patients;
6- To find if any specific body index and/or circumference perform better than the commonly used body mass index (BMI) as surrogate of BC in NAFLD patients.

1.4. Outline of this thesis

The present thesis is composed of ten chapters, as subsequently presented.

Chapter 1 – Introduction

The introduction presents the rational for the present investigation. The studied problem is disclosed together with the general research questions and purposes of the thesis. The thesis outline is presented and a mention to funding source is also made at the end of this chapter.

Chapter 2 – Review

This chapter presents a succinct review of the literature aiming to display the state of the art concerning the studied topics and to present the biological plausibility of the purposes of this research. This chapter is divided into three main parts. The first part focuses briefly on NAFLD epidemiology, pathophysiology and health consequences. The second part focuses on BC and fat distribution, its´ assessment and implications on health. The third part presents an overview of the ANS, including a general description of its' structure and function, cardiac regulation and respective interactions on metabolism, including BF regulations and other health and health risks implications.

Chapter 3 – Methods

This chapter provides a comprehensive description of the studied sample as well as the methods used for patients' assessment and data record. The inclusion of this chapter in the present thesis allowed the avoidance of the repetition of the description of the methodology used in each study, which was mostly similar, particular in respect to the studied sample and the dependent variables. This chapter firstly presents the "sample" and "study design" subsections to describe how the study was conducted, how the patients were recruited and the general sample characteristics,

and then presents the description of the methodology used for patients' assessment and data record, particularly BC and cardiac autonomic control.

Chapter 4 – Study 1: "Is Body Composition and Body Fat Distribution Related to Cardiac Autonomic Control in Non-Alcoholic Fatty Liver Disease Patients?"

This chapter presents study 1. The aim of this study was to determine if, and to what extent, specific markers of BC and BF distribution, are related with a selected mortality and cardiovascular risk related ANS marker in NAFLD patients. The association of different variables of whole and regional BC as well as ratios between different BF depots, used as BF distribution markers, with HRR, a known cardiac autonomic control marker reflective of parasympathetic reactivation, is analyzed.

Chapter 5 – Study 2: "Which is the Best Waist Circumference Measurement Protocol to use in Non-Alcoholic Fatty Liver Disease Patients?"

This chapter presents study 2. The aim of this study was to find which of the most used WC measurement protocols is preferable to be used in clinical practice with NAFLD patients. Four of the most used protocols to measure WC were tested according to different criteria in the quest of finding which seems best suitable for use in clinical settings with NAFLD patients.

Chapter 6 – Study 3: "Does the Wait Circumference Measurement Protocol Used Influences the Relation Between Waist-to-Height Ratio and Body Composition in Non-Alcoholic Fatty Liver Disease Patients?"

This chapter presents study 3. The aim of this study was to analyze whether the most used WC measurement protocols affect the strength of association between waist-to-height ratio (WHtR) and both, whole and central BF in NAFLD patients. In the literature WHtR was initially suggested using a specific WC measurement protocol (WCmp) however this ratio has been, most often, calculated using a different WCmp from the initially suggested. In this study four of the most used protocols to measure WC were used to calculate WHtR ratio and the results were compared in their association level with criterion whole and regional BC markers.

Chapter 7 – Study 4: "Does the Waist Circumference Measurement Protocol Used Influences the Relation Between Waist-to-Hip Ratio and Body Fat Content and Distribution in Non-Alcoholic Fatty Liver Disease Patients?"

This chapter presents study 4. The aim of this study was to analyze whether the most used WC measurement protocols affect the strength of association between waist-to-hip (WHR) ratio and BF content and distribution in NAFLD patients. In the literature WHR was initially suggested using WC measured at the minimal waist still this ratio has most often been calculated using a different WCmp from the initially suggested. In this study four of the most used protocols to measure WC were used

to calculate WHR ratio and the results were compared in their association level with criterion whole and regional BC and BF distribution markers.

Chapter 8 – Study 5: "Are Body Indexes and Circumferences useful as Surrogates of Body Fat content and distribution in Non-Alcoholic Fatty Liver Disease Patients as compared with the commonly used Body Mass Index?"

This chapter presents study 5. The aim of this study was twofold: (1) to analyze how body circumferences and indexes perform as surrogates of whole and regional BF content and BF distribution in NAFLD patients; (2) to find if any specific body index and/or circumference perform better than the commonly used BMI as surrogate of BC in NAFLD patients. The strength of associations between the clinical BC markers, comprising all studied body indexes and circumferences, and criterion BC and BF distribution dependent variables were tested. All correlation coefficients were compared to those obtained using BMI to identify alternative clinical markers.

Chapter 9 – Conclusions

This chapter presents the final conclusions of the present thesis. All studies are linked into one single research project and the main conclusions are presented in three separate sections: the first main conclusions concern the strength and limitations recognized in the overall research that is being presented; secondly the main findings of the present thesis are presented along with the respective practical implications; ultimately some recommendations and future directions of research are presented.

References

This chapter presents all the references cited in the present thesis, listed by order of appearance in the whole document. The references are numbered and cited according to Vancouver normative, which is more common in publications concerning biological and health sciences. The reason for the concentration of all references in the end of the document was to facilitate its' consultation, instead of spreading the references throughout the document at the end of each chapter.

1.5. Funding

This thesis was funded by the *Fundação para a Ciência e Tecnologia – Ministério da Educação e Ciência,* through individual doctoral scholarship (SFRH / BD / 41173 / 2007). This thesis was also integrated in a research project: *Parque de Ciência e Tecnologia do Alentejo - Laboratório de Investigação em Desporto e Saúde (Unidade de Fisiologia e Biomecânica – Composição Corporal e Sistema Nervoso Autónomo na regulação e Risco Cardiovascular)*, proposed by the Sport Sciences

School of Rio Maior (ESDRM) and by Health School of Santarém (ESSS) from the Polytechnic Institute of Santarém (IPS) and co-financed by national funds through the *Programa Operacional do Alentejo 2007-2013* (ALENT-07-0262-FEDER-001883), having Nuno Pimenta, MSc, as the researcher in charge.

Co-funded:

UNIÃO EUROPEIA
Fundo Europeu de Desenvolvimento Regional

QUADRO DE REFERÊNCIA ESTRATÉGICO NACIONAL
PORTUGAL 2007.2013

INALENTEJO 2007.2013

Chapter 2 – Review

"Concise review of the literature regarding Non-Alcoholic Fatty liver Disease, body composition and the autonomic nervous system."

The present chapter will present the state of the art concerning the studied topics, aims and instruments of the research being reported. This chapter is divided into three main parts. Firstly we will present a description of non-alcoholic fatty liver disease (NAFLD), its' pathophysiology, causes and consequences, and a characterization of the population diagnosed with this disease. Secondly we will make a review on body composition (BC) and body fat (BF) distribution, its´ assessment and implications on health. In the end we will present an overview of the autonomic nervous system (ANS), including a general description of it's structure and function, cardiac regulation and its' interactions on metabolism, including BF regulations and other health and health risks implications.

2.1. Non-Alcoholic Fatty Liver Disease

NAFLD is a rising metabolic condition that is under considerable discussion. Excess BF and particular BF accumulation patterns seem to be strongly linked to NAFLD and possibly play an important role in the disease aetiology, progression and consequences. This section outlines the pathophysiology and epidemiology of NAFLD, with a special focus on the importance specific metabolic disorders (insulin resistance and obesity) in the etiology of NAFLD.

2.1.1. General Overview of Non-Alcoholic Fatty Liver Disease

NAFLD is a rising recognized condition that has caught a growing focus over the last years (16, 26). The pathological picture of NAFLD resembles that of alcohol-induced liver disease, but it occurs in patients without an alcohol intake capable of injuring the liver (>20g/d for men and >30g/d for women) (16, 27-29). A diverse terminology has been used to characterize NAFLD, such as: fatty-liver hepatitis; nonalcoholic Laënnec's disease; diabetes hepatitis; alcohol-like liver disease and nonalcoholic steatohepatitis (16). The name non-alcoholic fatty liver disease has been considered preferable, encompassing the full spectrum of non-alcoholic, fat related, liver disease stages (16, 30).

There is not complete agreement on criteria for diagnosis of NAFLD, even though it has been suggested that it encompasses two basic histological lesions: (I) hepatic steatosis and (II) non-alcoholic steatohepatitis (NASH) (30-32). Hepatic steatosis consists of fat accumulation in hepatocytes mostly resulting from metabolic disorders (32). The degree of liver fat accumulation in NAFLD can be graded according to the percentage of hepatocytes with fat deposits: mild NAFLD involves less than 30% hepatocytes, moderate NAFLD up to 60%, and severe NAFLD above 60% (33), though a slightly different classification has been suggested by Brunt and colleagues (34). NASH is

characterized by hepatic inflammation and macrovisicular steatosis along with a constellation of other disturbances (32). The term NASH was introduced over three decades ago in the description of a group of Mayo Clinic patients, mostly obese and/or with diabetes mellitus, with an unnamed disease associated with a fatty liver histology, with elevated liver enzymes along with inflammation and fibrosis mimicking alcoholic hepatitis, but in the absence of alcohol intake (35). A proposal for grading and staging the histological lesions present in NASH was long presented by Brunt and colleagues (34). NASH can evolve to advanced fibrosis, cirrhosis and hepatocellular carcinoma (16, 30). Fat in the liver can be quantified by image methods [e.g. ultrasound of the liver has a high sensitivity and specificity, nearly 90%, for detection of fatty infiltration (36)] but the degree of inflammation and fibrosis, as well as other histological features of NASH can only be diagnosed by liver biopsy (16, 26, 37). Angulo and colleagues (38) have recently developed a scoring system to identify NAFLD patients with and without advanced fibrosis. By applying this model they were able to identify 90% of advance fibrosis using only routine measured and readily available clinical and laboratory data. In their study Angulo and colleagues (38) could have avoided 75% of performed liver biopsies. It is likely that other simple markers, strongly associated with NAFLD, will arise from research, and give significant information to estimate disease status.

The prevalence of NAFLD is estimated to range up to over 30% of general population in western countries, though accurate epidemiologic data are still not available in many countries (1-4). NASH occurs in up to 5.7% of the general population, in western countries (5, 39). Progression to cirrhosis can occur in 15 to 20% of NASH patients (40). In many countries more than 80% of NAFLD patients have an increased BMI and obesity has been found present in 25 up to nearly 60% of NAFLD patients (3, 41-44). Approximately 50% of NAFLD patients show signs of insulin resistance (IR) and 20-30% have type 2 diabetes, 80% present hyperlipidemia while arterial hypertension is present in about 30-60% (44). If we consider only the obese population, particularly in abdominal or in morbidly obesity, the prevalence NAFLD may range up to 100% (1-3, 26). Because of this strong association with obesity, mainly central obesity, and with impaired glucose metabolism, it is expected that NAFLD will strongly increase, establishing a parallel with the pandemic dimension of the mentioned metabolic disorders (6). Bottom line NAFLD is strongly associated to obesity, mostly that of the trunk (2, 16, 45, 46), to dyslipidemia, as mentioned before, mainly hypertriglyceridemia (39, 47), and to diabetes and IR (39, 46, 48), thereby NAFLD is also considered to be the hepatic manifestation of the metabolic syndrome (7, 41, 49). Given the close association between NAFLD and classical cardiovascular risk factors it is not surprising to find that patients with NAFLD have a higher

prevalence and risk of cardiovascular disease (CVD), as consistently shown by Targher and colleagues (8, 50-53).

2.1.2. NAFLD pathophysiology

NAFLD, in most cases, is a consequence of an imbalance between factors that promote liver fat increase (uptake and synthesis of fatty acids) and factors that promote liver fat reduction (secretion and oxidation of fatty acids) (16, 54). It has been suggested a "two-hit" hypothesis for the progression of NAFLD (55). The first hit, as mentioned, would be an imbalance in hepatic lipid metabolism favouring liver fat increase resulting in hepatic steatosis. The "Two-hit" hypothesis proposes that a second hit (oxidative stress and cytokine induction) is needed for inflammation to take place resulting in NASH (55, 56).

The increased level of lipids, mostly in the form of triglycerides, within hepatocytes in patients with NAFLD, results from an imbalance between the mechanisms that promote the uptake and synthesis of fatty acids and those that promote the oxidation and export of fatty acids (16). The accumulation of fat in the liver may be mediated by IR, regardless the fitness level of the patients (57). In fact, IR appears to be the most consistent explanation of the development of NAFLD (16, 32, 46). Two main mechanisms may be involved in the IR mediated hepatic fat accumulation: hyperinsulinemia and lipolysis (16). In healthy humans insulin stimulates adipocyte fat uptake and inhibits lipolysis (as explained in more detail in subsection 2.3.3.2 in this chapter), therefore reducing fatty acids flux from these cells. However, when cells present IR, insulin action is somewhat blunted and therefore lipolysis may be increased, resulting in increased fatty acid flux from adipocytes (58). The increased flux of fatty acids trough the portal vein towards the liver will result in high liver fatty acid uptake that will lead to mitochondrial β-oxidation overload, with the consequent accumulation of fatty acids within hepatocytes (16). IR initiates a compensatory effect by the pancreas β-cells that will increase insulin production leading to hyperinsulinemia (13) which in turn increases fatty acid synthesis in hepatocytes, by increasing glycolysis (32), and also favors the accumulation of fat within hepatocytes by decreasing the production of apolipoprotein 100, one main component of very low density lipoproteins (VLDL) (32, 59). In summary it is reasonable to assume that IR increases hepatocyte fat accumulation by favoring the hepatic metabolic pathways that promote lipid uptake and synthesis, limiting those that promote lipid degradation and secretion.

BC has an important role in the etiology of NAFLD (9) and might as well be a central cause of NAFLD. BF distribution may be even more crucial than whole BF in the etiology of NAFLD (5, 16).

Excess BF, observed in overweight and obese, besides being an important risk factor for developing NAFLD, is also strongly associated with IR and diabetes and may thereby contribute also indirectly for the development of NAFLD (5). The relation of increased BF with both IR and NAFLD, besides being a statistical relationship, has gained increased biological support. It has been observed that increased visceral adipocyte size is a strong predictor of increased triglycerides in the blood (60). More specifically, when visceral adipocytes are increased, they are predicted to contribute to nearly 50% of portal vein non-esterified fatty acids (NEFA) flux to the liver, as compared to only 5 to 10% observed in lean individuals (61). Together, this evidence supports the potential role of obesity, particularly abdominal obesity, in the development of excessive lipolysis related metabolic disorders, such as NAFLD. Moreover obese individuals, particularly the abdominal obese, display an adverse secretion pattern of several cytokines or adipokines, such as C-reactive protein (CRP), tumor necrosis factor-α (TNF- α), interleukin-6 (IL-6) and other which can alter lipolysis and insulin sensitivity (62-65), reproducing all the metabolic imbalances previously described to be associate to IR alone, that may lead to NAFLD. A quite recent study, conducted in rats, even showed significant elevation of hepatic triglycerides to precede the presence of IR (66). This is consistent with the assumption that obesity, particularly central obesity may be a key factor in NAFLD pathogenesis.

The so called “two hit” theory states that a second hit, after liver fat accumulation leading to simple liver steatosis (just discussed), is needed for NAFLD to progress to NASH (55). The second hit has been suggested to include mitochondrial dysfunction and lipid peroxidation (55, 56). Yet, mitochondrial dysfunction, quite recently, has been suggested to have a much more central role in the development of NAFLD, preceding hepatic fat accumulation (66) and therefore having a role in the development of steatosis (67) and this has been suggested to be somewhat linked to physical inactivity (68). The precise step that triggers simple steatosis to move to NASH is not completely understood. Visceral adipose tissue was found to be a risk factor for the presence of NASH, and may play a role (46). Mitochondria are the main cellular source of reactive oxygen species (ROS) and therefore have been suggested to be a major contributor to the progression of NAFLD (56). ROS are normal cellular end-products of oxygen metabolism, and are important signaling substances for redox balance or activation of defense mechanisms, however, high concentrations of ROS may be detrimental for cell metabolism and even for cell survival (69). As mentioned earlier hepatic steatosis occurs in the presence of mitochondrial β-oxidation overload, along with other metabolic impairments. Overloaded mitochondrial β-oxidation, present in steatotic hepatocytes generates increased opportunities for the formation of ROS which along with increased availability of fatty acid within hepatocytes, create an enriched cellular environment for lipid peroxidation. Lipid peroxidation

alters lipids integrity in a chain reaction way that may disrupt structural lipids (membrane lipids from organelles as mitochondria or from cell membrane itself) if not stopped (e.g. by anti-oxidants) (69). Peroxidation of mitochondrial structural lipids causes or increases mitochondrial dysfunction leading to higher formation of ROS, and vice-versa, increasing oxidative stress, which may end up causing hepatocyte apoptosis (programed cell death) or, in more adverse cases, necrosis (69). Ultimately these disturbances may lead to liver fibrosis and cirrhosis (55, 56, 70).

Bottom line, BC has an important role in the etiology of NAFLD (9). BF distribution may be even more essential than whole BF in the etiology of NAFLD (5, 16). Excess BF, observed in overweight and obese, and adverse BF distribution, besides being important risk factors that may contribute directly for developing NAFLD, are also strongly associated with IR and diabetes and may thereby contribute also indirectly for the development of NAFLD as well (5). Additional liver disturbances may be needed for NAFLD to progress to more adverse stages and outcomes (55).

2.2. Body Composition

The study of BC is strongly associated to, and sometimes confused for the study of obesity, though BC includes the study of body components far beyond fat mass alone. Nevertheless, in the present study, the focus will be mostly on BF content and distribution. BC is considered to be a component of health related fitness and it has proven to have physical, morphological and particularly important health implications (71, 72). In this section it will be presented a general overview of BC and its' health implications and a focus on BC assessment with some emphasis on BC clinical markers.

2.2.1. Body composition overview

The study of BC is a fascinating branch of the biological sciences. Imagining the alterations occurring in living body mass, from conception throughout lifespan cannot let anyone dispassionate. The field of BC research was suggested to be organized in three interconnecting areas: (I) BC levels and their organizational rules, (II) BC methodology, as well as (III) BC biological effects (73, 74). The first area involving the study, definition and links of the components themselves in each level of the suggested five-level model (described ahead in this subsection, see figure 2.1). The second area focuses on the study of BC measurement techniques to assess the various components in vivo. These include sophisticated laboratory methods as well as practical clinical methods and instruments. The third area involves the study of the influence of biological factors on BC, including those related to

both physiological and pathological conditions. Specific research topics included in this area of research comprises growth, development, aging, race, nutrition, hormonal effects, physical activity as well as diseases and medications that may influence BC. Even though this was not clear in the terminology proposed by Wang and colleagues for the organization of BC research, in the present thesis it was assumed that the influence of biological factors on BC (the third area of the proposed terminology) can be observed in both directions, meaning BC may be sometimes viewed as a determinant and/or as a consequence of abnormal or pathological conditions. Over the years a wide variation could be observed in the terminology and methodology used in the study of BC. The terminology and the five-level model suggested by Wang and colleagues (see figure 2.1) was milestone in the field of BC research and is still fairly consensual, despite the technology advances occurred. In the five-level model, it is assumed that each component within each level is mutually exclusive and the sum of all components in the same level is equivalent to whole body mass (73).

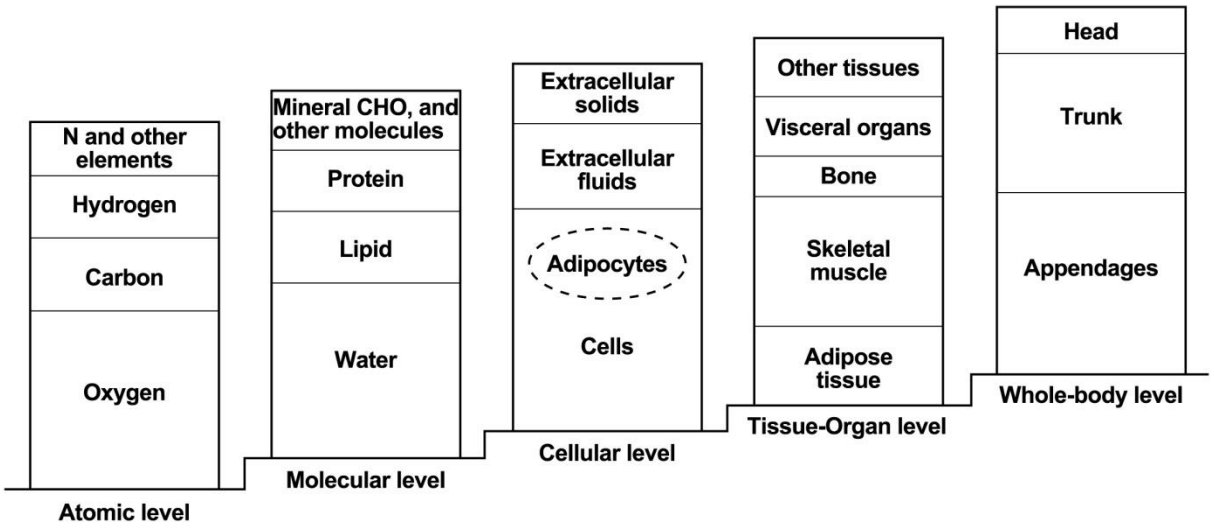

Figure 2.1 – The five-level model in body composition suggested by Wang and colleagues. Adapted from Shen and colleagues (74), pp 4. N: nitrogen; CHO: carbohydrates.

The atomic level includes eleven major components, including oxygen, hydrogen, carbon and nitrogen which all together account for more than 96% of whole body mass (74). The mentioned atoms constitute molecules including water, lipid, carbohydrates, proteins, lipids, bone minerals and soft tissue minerals, which are the six major components at the molecular level (74). This is probably one of the most considered level in BC analysis. At the molecular level it is possible to use multicomponent models: such as the widely used two compartment model (BF + fat free mass) which can be assessed by most methods available; or the three compartment model (eg. BF + bone mineral content + lean soft tissue) as can be assessed by dual energy x-ray absorptiometry (DXA); or the

common gold standard four compartment model (eg. Fat mass + total body water + total body protein + minerals) which needs to be assessed with a combination of methods (74). The cellular level includes three components: extracellular solids; extracellular fluids and cells, which include both fat and body cell mass. The tissue-organ level results from the differentiation of cells into tissues and organs, including adipose tissue, skeletal muscle, visceral organs and bone. At this level it is important to distinguish between adipose tissue and the wide studied BF or even from lipids, the latest both assessed at the molecular level (see figure 2.2) (74). Lipids include all of such molecules in the body, including non-fat lipids (lipids that are not in the form of triglycerides), both in adipose and other tissues, lipids stored within the adipose tissue in the form of triglycerides, and lipids in the form of triglycerides stored in other tissues. The commonly used word "fat" may be used interchangeably with BF and refers to triglycerides, which are the molecular form in which lipids are stored in the body. BF can be stored in the adipose tissue and in other tissues as well. Adipose tissue comprises BF and non-fat lipids, such as phospholipids, but includes also other components essential to the survival and function of adipose tissue cells. About 80% of adipose tissue is fat and the remainder 20% are water, proteins and minerals (75). The fifth level, the whole-body level, can be assessed using simple measures of weight or height, and can also be divided into regions such as the head, limbs and trunk, which can be assessed with various anthropometric techniques, such as circumferences, skinfolds and lengths.

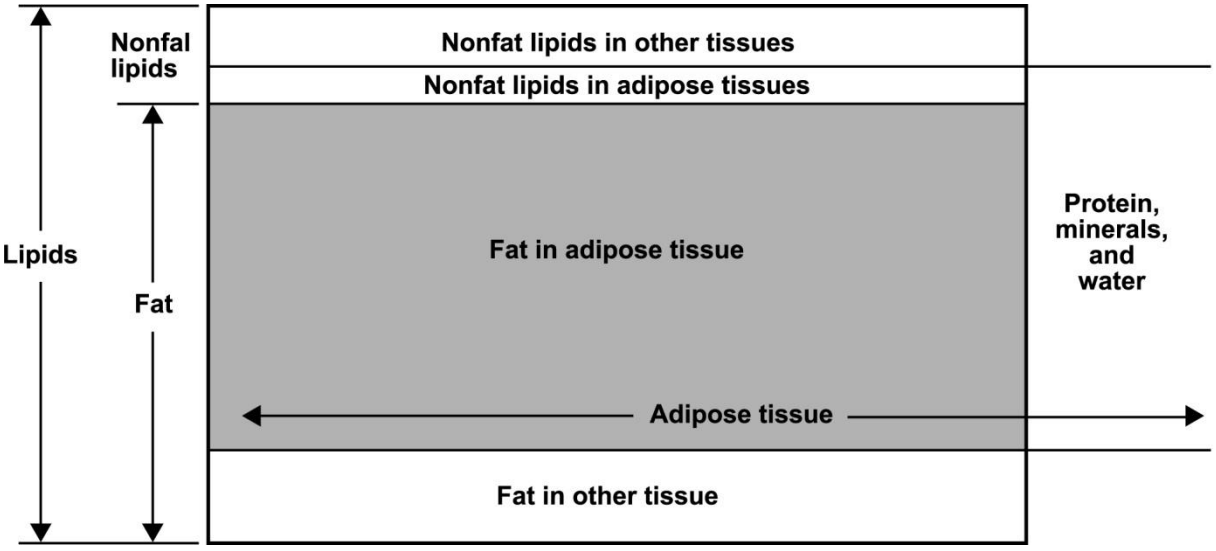

Figure 2.2 – The relationships between molecular-level components lipid and fat and the tissue-organ-level component adipose tissue. Adapted from Shen and colleagues (74), pp 12.

Shen and colleagues (75) presented a classification of adipose tissue location, based on image methods, which was very important to give some coherence to the wide terminology that was being used by then. In general adipose tissue is divided into subcutaneous and internal adipose

tissue. Subcutaneous may be divided into superficial and deep subcutaneous adipose tissue. Recently it has been given some importance to this division of subcutaneous adipose tissue (17). Internal adipose tissue can then be divided into visceral and non-visceral and these can subsequently be divided in many other specific fat depots, all according to their specific location (75). Sometimes BF may be stored in the visceral region, outside of adipocytes, near the organs. This is called ectopic fat and seems to have important health implications (76-78). Though this terminology was developed using image methods, DXA, despite being considered an image method, cannot assess all of the mentioned adipose tissues. DXA assesses BF in the whole body or body regions but cannot identify adipose tissue. Some attempts, however, were made to estimate visceral adipose tissue using DXA (79, 80) and found coefficients of correlation over 0.85, which is considered to be high. Yet DXA can only give precise estimates of whole BF, as does air displacement plethysmography (81), additionally DXA also estimates BF of specific regions or segments of the body, including the upper and lower limbs, the trunk or the abdominal regions, and other customized regions if intended (82). The present thesis adopts the terminology recommended by Sardinha and Teixeira (77): whole BF refers to whole body; regional BF represents a single variable of a body region, as in total abdominal fat; BF distribution refers to the measurement of one variable in relation to another so that a dichotomous fat distribution type can be identified (e.g., a contrast or a ratio), as is expressed in Vague's pioneer observations on this topic (83, 84). Never the less it is recognized that the expressions "regional BF" and "BF distribution" have been commonly used interchangeably (77).

BF distribution analysis started with the study of body shape (13). In the beginning of the 20th century, after World War II, insurance companies identified a higher risk of mortality related to certain BF distribution Phenotypes (11). In the conviction that fat accumulation in different regions of the body could have different predictive values, classifications of BF distribution were developed, such as Vague's classification (83, 84) that distinguished central BF accumulation (android) from peripheral, preferentially lower, BF accumulation (gynoid). BF distribution has long been shown to be related with other adverse outcomes, including diabetes, CVD, some forms of cancer and mortality (11). BF distribution markers have been suggested to be more consistent and strong predictors of CVD in healthy men and women, as compared to whole body markers of generalized adiposity (85), and the potential usefulness of such BF distribution clinical markers for public health has been recognized (86). BF distribution has also been shown to be particularly related to other metabolic impairments such as NAFLD (2, 16, 45, 46), as mentioned and explained in the previous subsection of the present work. More than six decades after Vague's preliminary publication, BF distribution is still receiving increased attention and related publications (17, 78). Image methods have been assumed

as criterion in the study of BF distribution, yet computerized axial tomography (CT) and magnetic resonance imaging (MRI), often used as reference methods to quantify adipose tissue (87, 88), do not assess BF (75). DXA assesses BC in a three compartment model within the molecular level and can estimate whole and regional BF content and distribution (82). Studies using DXA have however showed that BF from specific regions of interest (ROI) may be considered acceptable predictors of visceral adipose tissue (79, 80, 89, 90). Studies using ratios between DXA assessed ROI have also been able to predict MRI assessed visceral adipose tissue (91). BF can also be quantified by magnetic resonance spectroscopy (MRS) in various tissues-organs, such as the liver (92) or the muscle (93). Another image method often used to estimate liver fat content is ultrasound (94). Many clinical markers of BF content and distribution have been suggested, including simple anthropometric measurements and resulting calculated body indexes, as discussed ahead in the present chapter. These anthropometric variables and indexes assess BC at a whole-body level to evaluate body dimensions and morphology, and often are used as BF content and distribution surrogates.

2.2.2. Body Composition and obesity

The study of overweight and obesity has been overwhelming in the field of human BC research, maybe because of both the pandemic dimension of its prevalence in developed countries (95, 96) and the associated health implications of such widespread condition (14, 97, 98). The study of BC in specific subpopulations, such as NAFLD patients, is not so well explored as in the general population or in other specific subpopulations but is giving consistent steps and growing. Obesity has been defined as an excess of adipose tissue (71) however adipose tissue is difficult to assess, requiring expensive and limited access imaging methods such as computed tomography or magnetic resonance imaging (75). Obesity has also been referred to as an excess of BF (17, 99), which should be easier to estimate than adipose tissue. However the most widely used definition of obesity is “a state of excessive weight” mostly because of the simple and inexpensive marker recommended by most prominent organizations (72, 100-102) and most often used for the diagnosis of obesity: the body mass index (BMI = weight [kg] / height $[m]^2$). As mentioned, obesity is an important health concern and is linked to many other diseases and morbidities and ultimately, to higher mortality rates (11, 103), but the problem in obesity seem to be more related to altered metabolism of surplus fat and enlarged adipocytes, along with other metabolic impairments (17, 104, 105), rather than just an excess of body weight alone (106). Therefore whenever obesity is mentioned, in the present work, it should be referring to excess BF. The link between BF and related morbidities, or the detailed physiologic pathway that links them, are not still completely disclosed. Nevertheless it seems rather

consistent that the altered metabolism associated to excessive BF, either circulating fat as generalized intracellular fat, may play the pathological role in obesity (46, 104, 107-109). Obesity has been shown to be particularly linked to an increase in CVD, especially coronary artery disease, and this seemed to be mediated by other obesity related risk factors (e.g. diabetes, dyslipidemia, hypertension or sedentary lifestyle) (12, 15), however, in the Framingham Heart Study obesity was shown to be an independent risk factor of CVD, in both men and women (103, 110, 111), and in 1998 obesity was classified as a primary risk factor for CVD by the American Heart Association (112). Several excellent scientific reviews have been published focusing on the causes and health consequences of obesity, including all obesity related pathophysiological and pathogenic specificities (113-121). It is not the aim of the present work to replace these publications.

2.2.3. Clinical markers of body composition/obesity

This subsection focus on the clinical markers studied in the present thesis. This includes established and promising body indexes as well as body circumferences. A description and review regarding each studied clinical marker will be presented in the following subsections. To better organize the information, this subsection is divided in three parts: the first part regards the well-established BMI; the second part regards the selected body circumferences; the third part focus on body indexes, other than BMI.

2.2.3.1. Body mass index

BMI, calculated as weight, in kg, divided by squared height, in meters (BMI = weight [kg] / height $[m]^2$), is a simple marker of excess body weight, easy to measure, highly precise and strongly associated with overall fat (122) thus has been shown to explain 74% and 55% of the variation of whole absolute and relative BF (as assessed by Dual Energy X-ray Absorptiometry), respectively (123). BMI has also been shown to be particularly highly related with whole and subcutaneous adipose tissue, besides being strongly related with body circumferences and Waist-to-height ratio (WHtR) (discussed ahead in this subsection) (124). BMI has been massively used and endorsed by prominent institutions/organizations for the diagnosis of overweight and obesity (72, 100-102, 125). BMI however assesses BC at a whole body level, considering Wang's five-level model (73), and does not assess any specific BC component, though having, as mentioned, a significant association with generalized fatness (122). Therefore, when assessing obesity, BMI may be deceptive (17). The limitations of BMI have long been reported (21, 99, 126), several other markers have been advocated

(23, 127, 128), and new markers are being suggested regularly (17, 24, 129). Even though the usefulness of BMI may be accepted in the assessment of secular trends of the prevalence of obesity at the population level, BMI has been suggested to only provide a crude measurement of total adiposity and do not accurately diagnose obesity, at least at the individual level (126, 130). The endorsement of the use of BMI as a marker for obesity has relied recurrently on same arguments, including: BMI measurement is simple, non-invasive, reliable and inexpensive (131); BMI is associated with BC, particularly to fat (132), and BMI is also related with mortality and morbidity (133-136), including with the prevalence of NAFLD (137). The first argument may be very well applied to other markers, such as WC, which has been suggested to be as much or even more simple, non-invasive, reliable and inexpensive as compared to BMI (23). The second and third arguments have also shown fragilities and are somewhat interrelated (21). The BMI has been shown to be highly related with whole BF assessed by DXA (138), which is the most important body component in determining obesity, and has even been shown to be associated with abdominal BF depots, particularly subcutaneous adipose tissue (139). However BMI is also related to other body components of the lean tissue, such as muscle or bone (21). Ultimately a person with high levels of muscle mass and bone mineral density may be classified as overweight or obese, even if he/she does not have excess BF or related metabolic disorders. On the opposite direction, subjects with normal BMIs are often shown to have excess BF and related metabolic disorders. Individuals with the later mentioned phenotype are often referred to as metabolically obese normal-weight individuals (45). BMI does not take into account the heterogeneity of regional BF deposition (140), which has been identified as an important correlate leading to CVD (13) and possibly to NAFLD (5, 16). Frankenfield and colleagues (99) found that 30% of men and 46% of women with BMI under 30kg/m^2 had obesity levels of fat, as defined by results over 25% or 30% of BF for men and women respectively. The under prediction of obesity has been considered a greater error than its over prediction because the risk is higher for the development of co-morbidities in under predicted obese patients, which may also skip or delay important therapy (126). In specific subpopulations, such as CVD patients (141), dialysis patients (142) or even elder individuals (143), increasing BMI seems to be protective, which is completely against the concept of obesity. This underlies the concept of the “obesity paradox”, firstly introduced over ten years ago by Gruberg and colleagues (144) and was further studied and consolidated, particularly by Lavie and colleagues (145-164). A fairly recent report by Coutinho and colleagues (141) helped enlighten this topic and should hopefully spur further studies to consider going beyond the BMI to assess the morbidity/mortality risk associated with excess adiposity in specific subpopulations such as that of patients with CVD. The mentioned report provides robust evidence that anthropometric correlates of body shape, such as WC and the WHR, matter a lot more

than the BMI, which is only an index of relative body size and not of BC /BF distribution, in the subpopulation of CVD patients. The recent study by the same research group (165) showed that the highest mortality risk in CVD patients was found in subjects with a BMI of 22kg/m^2 and a WHR of 0.98, which means a normal weight person with central fat accumulation. This may lead to the assumption suggested by Despres, that the so called "obesity paradox" may rather be a "BMI paradox" (130). Other subpopulations should be tested for the usefulness of BMI and alternative tools should be standardized at least when BMI proves deceptive.

2.2.3.2. Body circumferences

Over the years a considerable amount of tools and markers have been advocated for the assessment of BC and the associated health risks, either alternatively and/or complimentary to BMI. In the present work some of the most conspicuous proposals were studied. Body circumferences have been shown to be less skill dependent and have lower inter-observer variation, as compared with skinfold measurement (166, 167). Body circumferences, sometimes called body girths (168), have been also widely used and recommended (23, 72, 86, 102, 125, 167, 169-173), for use in clinical settings. WC measurement has been extensively used in different settings and populations (23, 86, 169), including the subpopulation of NAFLD patients (174). WC is considered a risk factor for NAFLD (175, 176) and was found to be related with CVD related morbidity, with diabetes and with cardiovascular as well as all-cause mortality irrespectively of WC measuring site (23, 177, 178). WC cut off values to assess overweight and obesity, as well as increased cardiovascular risk, have been developed for different ethnic groups (179), however the most commonly used cutoffs are those defined by Lean and colleagues (180, 181) (overweight = 94 cm and 80 cm; obesity = 102 cm and 88 cm; for men and women, respectively), derived from a cross-sectional population predominantly from European origin, yet worldwide accepted cut off values are yet upcoming (86). Specific WC cut off values have been developed for Japanese workers (85 cm for men and 80 cm for women) above which was recommended that everyone should be referred for fatty liver appraisal through abdominal ultrasound (182). In NAFLD patients WC has been found to be associated with several metabolic impairments including IR (183, 184) as well as liver fat (174) and NAFLD severity (185, 186). Moreover high WC was also found to be related to increased health-care costs (187). WC has been considered a proper surrogate of BC, particularly when focusing on regional BF or in BF distribution, in the general population and in selected subpopulations (139, 169, 188), showing high correlations with most body circumferences, particularly with arm and hip circumferences (hip-C) (r^2=0.89 and 0.84, respectively), and also with visceral, subcutaneous and whole adipose tissues, in addition to

both BMI and WHtR (124). Observations using computed tomography showed that WC was better predictor than both BMI and WHR, of overall abdominal BF depots, including intraperitoneal and retroperitoneal adipose tissues plus anterior and posterior abdominal subcutaneous adipose tissues (139). WC was also found a very good predictor of both trunk and waist BF, as assessed by DXA (138). Conversely, the relation of WC with NAFLD patients' BF content and distribution has been overlooked. Also, even with the robust evidence concerning WC as a strong BC marker and a predictor of the risk of numerous diseases and adverse outcomes, and also despite the widespread usage of WC, there is currently no optimal and uniquely recommended WC measurement protocol (WCmp) to be used in clinical practice, either in general population as in specific higher risk subpopulations. Several WCmp have been recommended by sound authorities, such as the World Health Organization (WHO) (125) or the International Society for the Advancement of Kineanthropometry (ISAK) (168), but scientific rational is lacking to recommend one single protocol (23, 189). The suggested protocols differ mainly on the anatomical landmarks and correspondent measuring sites (see table 2.1). The most frequent WC measurement sites found in the literature were at the midpoint between the lowest rib and the iliac crest, as suggested by the WHO, at the minimal waist (as suggested by ISAK) and at the umbilicus, still a fourth measurement site has also been used and endorsed by the National Institute of Health of the United States and also by the Canadian Society of Exercise Physiology, which is measured just above the iliac crest (23, 102, 171, 190). Nevertheless several other measuring sites have been sparsely used (23).

Table 2.1 – Waist circumference measurement landmarks and references.

References	Protocol
ISAK (168); ASRM (170); ACSM (72)	Measured at the level of the narrowest site of the torso (minimal waist).
NIH/CDC (171, 190); CSEP (189)	Measured right above the iliac crest.
WHO (86, 125, 191)	Measured at the mid-distance between the last rib and the top of the iliac crest.
Commonly used, including in studies on NAFLD (51, 68)	Measured at the level of the umbilicus.

ISAK – The International Society for the Advancement of Kinanthropometry; ASRM – Anthropometric Standardization Reference Manual; ACSM – American College of Sports Medicine; NIH – National Institute of Health; CDC – Centers for Disease Control and Prevention; CSEP – Canadian Society of Exercise Physiology; WHO – World Health Organization.

Besides WC, other body circumferences have been used and suggested for the study of human morphology and BC (168, 170-173), yet the volume of the knowledge and publications concerning these other body circumferences are not comparable to that of WC. Unlike WC measurement protocol, the protocol for hip-C measurement, at the maximum extension of the buttocks, is fairly consensual (168, 170-172). Smaller hip-C seems to be associated with an adverse metabolic profile, including increased features of the metabolic syndrome (192) and with higher

intima-media thickness (193), in obese women. Still small hip-C seem to decrease survival in both men and women, however physical activity may counterbalance, at least partially, the adverse effect of small hips (194). Larger hip-C, conversely, were shown to be associated with a lower risk of type 2 diabetes, lower glycemia and better lipid profiles, independently of BMI, age, and WC (195-199). These seem to be true particularly for women, when adjusting for WC (200, 201). This inverse association of larger hip-C with diabetogenic and atherotrombotic profile, for any given WC, was confirmed in our laboratory, in obese women (202). Ethnicity was argued to be irrelevant in the inverse relation between hip-C and hazardous outcomes (196), however, opposite results have also been found in specific populations, such as the Australian aborigines and Chinese (203, 204). In a recent cohort of both men and women, first degree relatives of diabetic patients, hip-C was neither a positive nor negative risk factor for incidence of diabetes (205). A recent meta-analysis by the same authors, however, confirmed the inverse relationship between hip-C and risk of type 2 diabetes in both men and women (206). Also quite recently, hip-C was found important for the assessment of mortality risk and it was suggested that central obesity related risk, as assessed by WC, may be seriously underestimated if hip-C is not taken into account (207). Hip-C was found among the highest correlates for the percentage of whole BF, as well as for BMI and WC (24). Hip-C has been shown to be associated with subcutaneous adipose tissue and, to a much lesser extent, with skeletal muscle and with visceral adipose tissue, in both men and women, as opposed to WC which seems more related to visceral adipose tissue (124, 208). Hip-C was also found to be correlated with the other body circumferences as well as with BMI and WHtR (124). Hip-C correlates in NAFLD patients are unknown. A model for calculating BMI based on WC and Hip-C measurements was developed with promising results, meaning whenever circumference measurements are available, the measurement of both weight and height to calculate of BMI may be redundant (209). Recently the measurement of both waist and hip-C were recommended to be considered together, but not as a ratio, to improve risk prediction models for cardiovascular and other hazardous outcomes (210).

Another common body circumference described in the literature is the thigh circumference (thigh-C). Thigh-C measurement protocol, though not as inconsistent as WC measurement protocol, is also not as consensual as the protocol for hip-C measurement, as there are varying terminology and references for its assessment. In a milestone publication launched over two decades ago, thigh-C measurement was suggested in three different ways, each using its' own landmarks and measuring sites: proximal thigh-C; mid-thigh-C and distal thigh-C (170). Most authors and institutions/organizations used and endorsed mid-thigh-C measurement (167, 172) also called mid-thigh girth (168) or simply thigh-C (173) as will be called throughout the present work. Smaller thigh-

C were shown to be associated with increased risk of CVD, IR and premature death (211-213). As with hip-C, larger thigh-C were shown to be associated with a lower risk of type 2 diabetes, independently of BMI, age, and WC (195). Thigh-C was shown to be associated to subcutaneous adipose tissue, and seem to have also a moderate relation with skeletal muscle and whole adipose tissue, though being associated with other body circumferences (124, 208). Associations with whole BF were also found in girls (214). Thigh-C has also been shown a strong association with both BMI and WHtR, though not as strong as that obtained between other body circumferences and the same body indexes (124).

Arm circumference (arm-C) measurement, occasionally called arm relaxed girth (168) or mid-upper arm-C (172, 215) or even mid-arm-C (216, 217), has been described according to two mainstream measurement protocols. One arm-C measurement protocol was standardized about 25 years ago, as described in the Anthropometric Standardization Reference Manual (170), is widely used in the United States of America and is endorsed by the National Institutes of Health (171, 190) and others (167, 172, 173), which is measured at the mid distance between the acromion and the olecranon, measured with the elbow in a 90^{o} flexed position. The other prominent arm-C measurement protocol soundly endorsed by the ISAK (168) is measured at the mid-distance between the acromiale and the radiale landmarks, with the arms hanging by the sides in a relaxed position. Arm-C has been used for decades in the assessment of malnutrition in diverse populations, including children and underdeveloped countries population, particularly in emergency settings (215, 218-221). Also for some decades now arm-C has also been known to influence blood pressure measurement and the diagnosis of hypertension (216, 222-224). Arm-C was later found to be positively correlated to hypertension related cardiac left ventricle abnormalities, yet showing a small effect size (225). Low arm-C was associated to poor prognosis in advanced cancer patients (226) and seems a better predictor of long term mortality risk, as compared to BMI, in older adults (220, 227). Arm-C seems highly reproducible and related with body weight, therefore it has been recommended that it should be used more often in nutritional studies (217, 228). It has been shown to be a good measure, capable of detecting variations as small as 2.2%, particularly when assessed always by the same observer (229). Arm-C seems particularly related with skeletal muscle, in addition to subcutaneous and whole adipose tissue, and was found to be related also with the other body circumferences, as well as with both BMI and WHtR (124). Arm-C correlates in NAFLD are unknown.

Also occasionally used is calf circumference (calf-C), also called calf girth (168), which measures the widest circumference of the leg. Calf-C has been widely used in India for the

assessment of low birth weight (230-235). Calf-C has also been used to assess Duchenne dystrophy, which is characterized by increased calf volume, despite progressive muscle weakness (236). In elderly individuals low calf-C has been related to muscle disability, limited physical function (237) as well as nutritional status (238-241). An inverse association was found between calf-C and atherosclerosis, suggesting a protective effect of increasing calf-C, but this was only observed in elderly (242) and in type 2 diabetes Korean patients (243). Nevertheless calf-C seems to be positively related to insulin sensitivity in type 2 diabetes Korean patients (213). Low calf-C was found somewhat predictive of mortality in elderly individuals (177, 227, 244). Calf-C was found to be particularly related with skeletal muscle, in addition to subcutaneous and whole adipose tissue (124). Calf-C also showed strong correlations with the other body circumferences as well as with both BMI and WHtR (124). Calf-C correlates in NAFLD are unknown.

2.2.3.3. Alternative body indexes

In addition to BMI and single anthropometric measurements, other composed variables, often called indexes, have been developed and have also been shown to be of value in the assessment of BC, BF distribution and related health risks (24, 91, 129, 138, 245). One of such body indexes was the WHR, calculated as WC divided by hip-C, both in centimeters (WHR = WC [cm] / HC [cm]). WHR has been suggested mainly as a BF distribution surrogate and has been used to predict BF distribution related risk (86). WHR has been consistently linked to metabolic disorders, including type 2 diabetes, hypertension and dyslipidemia, in diverse populations (178, 246-249) and to the respective outcomes, particularly cardiovascular (247, 250, 251), and was also shown to be closely related to the occurrence of NAFLD (252). Increased WHR has also been linked to a higher specific, particularly cardiovascular, and all-cause mortality (141, 253-258) however opposite results have also been found (259, 260). Still cut off values of 0.95 for men and 0.80 for women were defined for the identification of high WHR (261). WHR was found positively correlated with intraperitoneal and retroperitoneal adipose tissues as well as with anterior and posterior abdominal subcutaneous adipose tissues, yet WC performed better than WHR in predicting the later (139). Plus WHR was found to be moderately related with DXA derived BF ratios, including trunk BF-to-total BF ratio and trunk BF-to-appendicular BF ratio (262). Though, strong association have also been found between WHR and both DXA assessed trunk BF-to-appendicular BF ratio and waist BF-to-hip BF ratio (138). WHR correlates in NAFLD patients needs further attention. Even though WHR was initially suggested to be calculated using measurements of WC at the minimal waist (261), the WC measured at the mid distance between lowest rib and iliac crest has been the most used protocol (179). The considerable

variation in methodology and results found in the literature may be limiting, to some extent, a wider usage of this body index, however it is not known if this influences WHR correlation with BC, BF distribution and related health risk. WHR is assumed to reflect the distribution of fat throughout the body and a high WHR should represent a preferential abdominal or central accumulation of BF (128) and it has generally proven to be so (91, 138, 139) yet conflicting results have also been found (263). The importance of using one consensual standardized measurement protocol to calculate WHR was soon identified (264, 265). Yet no single consensual measurement protocol in recognized and a wide diversity of methods for the measurement and calculus of WHR can be found in the literature (179).

Another promising body index is the waist-to-height ratio (WHtR), calculated as WC divided by height, both in centimeters (WHtR = WC [cm] / height [cm]) (129). WHtR is an index of abdominal obesity initially suggested by Hsieh and Yoshinaga in the mid-nineties (266, 267). By then WHtR was suggested to be a better predictor of multiple coronary heart disease risk factors than other obesity and fat distribution indexes in both men (267) and women (266). A cut of value of 0.5 was suggested for the diagnosis of high WHtR in both male and female individuals from different ethnic groups (245, 268, 269). This cutoff value has been argued to support a strong public health message: “keep your waist to less than half your height”! Heymsfield and colleagues (124) presented data providing the conceptual foundation, based on a geometrical model, to support the relation of body circumferences with body size and composition, specifically in relation to height, which is the rational for WHtR. Additionally WHtR has been suggested to be preferable to other indexes and clinical assessments, including BMI, WC and WHR, to predict individual and clustering cardiovascular risk factors, including diabetes, hypertension and lipidemia, in different ethnic and age groups (129, 270-274) tough comparable results have also been found (247, 270, 275) particularly with WC (276). Besides being associated with traditional cardiovascular risk factors high WHtR has been shown to be associated uric acid, C-reactive protein, and liver function enzymes (aminotransferases) (277). WHtR was also considered marginally preferable to BMI, WC and WHR in the longitudinal identification of incidence of CVD and related events (247) as well as to all-cause mortality (254). WHtR has been advocated to be a good alternative to BMI in the assessment of obesity related risk, and strong arguments have been putted out, including the fact that it is more sensitive than BMI as an early warning of health risk; it is cheaper and easier to calculate as compared to BMI; the cutoff value of 0.5 may be used in males and females from different ethnic and age groups, including children, which is not true for BMI (269). To our knowledge only one study focused on NAFLD patients using WHtR (252). WHtR seems elevated in NAFLD patients but was not a better prognostic factor of NAFLD than were BMI or WHR. Additional research concerning in NAFLD patients is needed to establish

unequivocal usefulness of WHtR in this sub-population. WHtR has been studied mainly in relation to metabolic abnormalities and cardiovascular risk however some observations have also showed WHtR to be at least similarly associated to abdominal fat as is WC, and better than both BMI and WHR (278, 279). WHtR was found to be more associated to visceral than to subcutaneous adipose tissue, as assessed by MRI (91, 275) however higher correlations with subcutaneous and whole adipose tissue was also found (124). WHtR was shown to be related with overall body circumferences, as well as with BMI (124). Despite some rather robust body of evidence regarding the study of WHtR, there is still some inconsistency considering the WCmp used to calculate WHtR (280). Scientific rational is lacking to recommend one single WCmp among the several protocols that have been suggested and endorsed by sound authorities, and used by prominent authors (23, 169, 189). Using different WCmp result in different WC magnitudes and therefore are not interchangeable (23). WHtR was initially suggested using WC measured at the umbilicus (266, 267). In a recent review (280) on WHtR, WC measured midpoint between the lowest rib and iliac crest was found to be used in 50% of the reviewed papers, and for that reason its routine use was encouraged.

A novel body index was suggested rather recently, and claimed to be a better predictor of whole body adiposity than other already established common body indexes (24). Yet conflicting results were promptly disclosed (281) and have been reported since (25, 282, 283). The body adiposity index (BAI), calculated as the hip-C, in centimeters, divided by height, in meters, to the 1.5 power minus eighteen (BAI = (Hip-C [cm] / height [m] $^{1.5}$) – 18), was shown to predict percentage of BF (%BF), as assessed by DXA, with highly concordance (24), and it has generally proved to do so, in different populations (284, 285). Some studies however countered the argument that BAI is superior to other indexes, such as the BMI (25, 281), but also this counterargument has not been consistent (286). Of notice is the fact that hip-C measurement protocol is quite standardized and therefore this body index does not have the limitation reported for WHR and WHtR, which is the inconsistency regarding WCmp. BAI, though seemingly predictor of %BF, has been suggested to be ineffective to cardiovascular risk (282, 287-289). This is not surprising if taken into account the available information concerning the link between hip-C, a key component of BAI, and cardiovascular risk (described earlier in this subsection). Accordingly BAI was found to be inferior to BMI in the prediction of most cardiovascular risk factors (275, 290, 291). No studies could be found, using BAI, neither related to NAFLD, nor conducted in NAFLD patients.

2.3. Autonomic Nervous System

The human nervous system comprises an ensemble of structures and a load of functions, many of them still under discussion. The autonomic nervous system is considered to be an important part of the human nervous system that strongly contributes for the dynamic maintenance of body's homeostasis. A general overview of the human nervous system and a specification of the anatomy and functions of the ANS, particularly those related with lipid metabolism with possible important implications in body composition, will be presented here.

2.3.1. General overview of the human nervous system

The Human nervous system comprises two main groups of structures with different functions: the central nervous system (encompassing the brain and the spinal cord) and the peripheral nervous system (including the efferent and the afferent divisions) (292). The ANS is part of the efferent division of the peripheral nervous system and is involved in the innervation of all tissues other than skeletal muscle (292). The ANS includes the enteric nervous system as well as both the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). The enteric nervous system is a specialized nerve network in the wall of the intestinal tract and will not be further discussed here.

2.3.2. The Autonomic Nervous System Divisions

Both SNS and PNS have some similitudes, e.g. both have two-neuron connections between central nervous system and the effector cells, the first neuron has its cell body in the central nervous system and ends in an autonomic ganglion were it synapses with a second neuron that leads the stimulus to the effector cell or organ (292). However there are important differences between both SNS and PNS that makes possible for these two structures to have such distinct and unique functions. One such difference is in the communication with effector cells and organs: in the synapse between the first neuron (preganglionic fiber) and the second neuron (postganglionic fiber), both SNS and PNS use mainly the same neurotransmitter which is acetylcholine, however, in the SNS, the major neurotransmitter between the post ganglionic fiber and the effector cells is norepinephrine while in the PNS it is still acetylcholine (292). This underlies one major difference between both ANS divisions, that is crucial for the distinct, many times opposite, effects exerted in effector cells and related organs. Another important difference relies on the anatomical arrangements of both divisions: SNS, to some extent, has an entire system tied together so that it can act as a single unit, despite some small independent regulations; PNS, in contrast, is made up of relatively independent components, making it possible to have more variable responses, more tailored to specific demands (292). One

last difference discussed here is that SNS has two input pathways to target effector cells and organs: (I) a normal neuronal pathway through its' neurotransmitter (norepinephrine) released by postganglionic fibers and (II) an adrenergic pathway through the adrenal medulla which is a set of postganglionic specialized neurons that never develops axons, instead, when activated by preganglionic fibers, release epinephrine (about 80%), norepinephrine, plus small amounts of other substances as dopamine and ATP, directly to the bloodstream (292). These substances, called hormones rather than neurotransmitters, are them transported via the blood to the effector cells and organs that may or may not be near SNS neurons and still be activated by circulating epinephrine and norepinephrine. The PNS only reaches effector cells and organs through its' postganglionic fibers.

The ANS is controlled by the central nervous system however the regulation of autonomic functions is highly dependent on visceral sensory feedback conveyed from the receptors located in overall organs to the central nervous system including a network of visceral sensory neurons which constitute the central autonomic network (CAN) (293). The CAN helps controlling and is affected by several physiologic factors such as blood pressure, respiration or the circadian cycle and has a tonic, reflex and adaptive control over autonomic (294). A description of the CAN components and function, particularly concerning cardiac autonomic control, is well summarized elsewhere (294, 295). Because the activity of both divisions of the ANS is somewhat controlled by the CAN, disorders involving the CAN may manifest themselves as autonomic hyperactivity or as autonomic failure resulting in abnormal outcomes in different organs and bodily functions leading to metabolic disorders such as obesity or essential hypertension, depending on in each direction and each of the ANS division is most altered (294).

2.3.3. Functions of the autonomic nervous system

This section will discuss ANS functions, particularly those related to cardiac control and metabolic control, especially lipid and adipocyte metabolism. The ANS has many functions that concur for homeostasis (also known as dynamic constancy) including smooth muscle and cardiac regulation and secretory gland control (292, 293). The SNS and the PNS have different, sometimes opposing, functions in the body. Generally the SNS increases body's response under conditions of stress (e.g. exercise), increases energy expenditure generating a so called "catabolic state" (fight-or-flight response) whereas the PNS is dominant in resting vegetative functions, promotes energy storage supporting a typically "anabolic state" (rest-or-digest state) (293). In the present document the focus will be mainly on cardiac and adipocyte autonomic regulation.

2.3.3.1. Cardiac autonomic control

This section centers on the control of heart function by the ANS, particularly that related to exercise, and the consequent recovery from such physiological challenge. The ANS is involved in cardiac autonomic control at rest and in response to challenges such as orthostasis, thermic regulation or exercise (293, 295). PNS exerts an inhibitory effect, through the vagal nerve, which reduces heart rate and ventricular contractility so that the heart is not exposed to excessive unnecessary work (20). At rest PNS is dominant in the control of cardiac function, which causes sinoatrial node to decrease heart rate (292). In the complete absence of any influence, neither hormonal nor neural, the sinoatrial node pace is about 100 beats per minute, however, at rest most humans have lower heart rates due to PNS action (292). At the onset of exercise, many times even before the start of actual exercise, heart rate increases in response to decreases in PNS activity to the vagal nerve, affecting the sinoatrial node and ventricular muscle of the heart, which reduces the tonic suppressive effect of the vagal cardiac stimulation on heart rate and ventricular contractility (20). Subsequent and complementary activation of the SNS nerves to the heart plus the SNS stimulated released of epinephrine in the adrenal medulla, to the blood, will result in additional increases in heart rate and ventricular contractility during exercise, primarily via stimulation of B-adrenergic receptors in the heart both by neural (mostly norepinephrine) and endocrine (mostly epinephrine) catecholamines (20, 292). In summary, heart rate increases in response to exercise through a combined action of SNS and PNS. At exercise cessation the increased heart rate rapidly decreases throughout exercise recovery. This rapid recovery is mediated mainly by PNS reactivation and seems to be important in reducing excessive cardiac work after exercise (296).

Specific disturbances in the ANS have been identified as strong risk factors for cardiac disease, morbidity and mortality (297-302). The relation of ANS and mortality was initially hypothesized by Schwartz and colleagues (303). Since the findings by Imai and colleagues (296) suggesting early heart rate recovery (HRR) as a parasympathetic reactivation marker, as later confirmed (304), several studies have shown poor parasympathetic cardiac control, as assessed by HRR, to be strong and independently related to higher risk of mortality and other hazardous cardiovascular and metabolic outcomes, either in general population referred for symptom limited graded exercise test (297-299, 305-307) as in patients with established cardiovascular (308, 309) or metabolic diseases (18, 310, 311) (table 2.2).

Table 2.2 – Studies examining the usefulness of heart rate recovery as a prognostic marker.

Reference	Setting	Purpose	Sample	Exercise protocol	HRR protocol	HRR Cutoff values	Main conclusions
Cole et al. (299)	Cleaveland Clinic Foundation.	Examine the usefulness of HRR as a prognostic marker of overall **mortality**.	n = 2428 Adults referred to for a first symptom limited test.	Standard and modified Bruce Protocols.	HR recorded at maximum exercise and at 1 minute of recovery; Recovery: - In treadmill; - 1.5 mph; - 2.5% incline.	Low HRR1' ≤ 12bpm	A low HRR after maximal exercise is a powerful and independent predictor of the risk of death.
Nishime et al. (305)	Cleaveland Clinic Foundation.	To examine the importance of SPECT perfusion abnormalities relative to functional capacity and HRR for prediction of all-cause **mortality**.	n = 9554 Adults ≥30yr Consecutive patients referred specifically for exercise ECG.	Standard and modified Bruce Protocols.	HR recorded at maximum exercise and at 1 minute of recovery; Recovery: - In treadmill; - 1.5 mph; - 2.5% incline.	Low HRR1' ≤ 12bpm	HRR is an independent predictor of mortality.
Shetler et al. (312)	Veterans Affairs Palo Alto Medical Centers.	To validate HRR as prognostic tool for all-cause **mortality**, evaluate its diagnostic value for coronary artery disease and clarify some of the methodological issues surrounding its use.	n = 2193 males (age = 59±10yr) Patients who underwent exercise testing and coronary angiography between 1987 and 1998, without cardiac disease.	Treadmill protocol from the U.S. Air Force School of Aerospace Medicine Or an Individualized Treadmill ramp protocol.	HR recorded at maximum exercise and at 1, 2 3 and 5 minutes of recovery; Recovery: - passive recovery; - In supine position.	Low HRR2' ≤ 22bpm	HRR at 2 minutes had prognostic value for all-cause mortality but not diagnostic value for coronary artery disease.
Diaz et al. (308)	Cleaveland Clinic Foundation.	To examine the importance of SPECT perfusion abnormalities relative to functional capacity and HRR for prediction of all-cause **mortality**.	n = 7163 (25% women). Adults ≥ 30yr. Consecutive patients referred for symptom-limited exercise testing with SPECT imaging.	Standard and modified Bruce Protocols.	HR recorded at maximum exercise and at 1 minute of recovery; Recovery: - In treadmill; - 1.5 mph; - 2.5% incline.	Low HRR1' ≤ 12bpm	Myocardial perfusion defects independently predict all-cause death, even after accounting HRR and other potential confounders.

HRR – heart rate recovery; HR – heart rate; SPEC - thallium201 single photon emission computed tomography; ECG – Electrocardiogram; CAD – coronary artery disease; CVD – cardiovascular disease; DM – diabetes mellitus; CV – cardiovascular; T2DM – type 2 diabetes mellitus; CHD – coronary heart disease.

Table 2.2 – Studies examining the usefulness of HRR as a prognostic marker (continuation).

Reference	Setting	Purpose	Sample	Exercise protocol	HRR protocol	HRR Cutoff values	Main conclusions
Watanabe et al. (306)	Cleaveland Clinic Foundation.	To determine whether the predictive value for **mortality** of HRR persisted even after considering estimated left ventricular ejection fraction.	n = 5438 Adults ≥ 30yr. Consecutive patients referred for exercise stress echocardiography	Standard and modified Bruce Protocols.	HR recorded at maximum exercise and at 1 minute of recovery; Recovery: - passive recovery; - In supine position.	Low HRR1' ≤ 18bpm with stress echocardiography.	HRR is a powerful and independent predictor of death, even after accounting for left ventricular systolic function.
Vivekananthan et al. (307)	Cleaveland Clinic Foundation.	To determine whether abnormal HRR predicts **mortality** independent of the angiographic severity of coronary disease.	n = 7163 (25% women). Consecutive patients ≥ 30yr, referred for symptom-limited exercise testing with SPECT imaging.	Standard and modified Bruce Protocols.	HR recorded at maximum exercise and at 1 minute of recovery; Recovery: - 1 minute; - In treadmill; - 1.5 mph; - 2.5% incline.	Low HRR1' ≤ 12bpm Or Low HRR1' ≤ 18bpm with stress echocardiography.	HRR is independently predictive of mortality, even when controlled for angiographic severity of CAD, left ventricular function, and exercise capacity.
Lipinski et al. (298)	Veterans Affairs Palo Alto Medical Centers.	To determine whether abnormal HRR predicts **mortality** independent of the angiographic severity of coronary disease.	n = 2193 men Patients who underwent exercise testing and coronary angiography between 1987 and 1998, without cardiac disease.	Treadmill protocol from the U.S. Air Force School of Aerospace Medicine Or an Individualized Treadmill ramp protocol.	HR recorded at maximum exercise and at 1, 2 3 and 5 minutes of recovery; Recovery: - passive recovery; - In supine position.	Low HRR1' ≤ 12bpm and Low HRR2' ≤ 22bpm	Decreased HRR at 2 minutes, is a strong predictor of mortality independent of other variables, and predicts also the presence of CAD.
Cheng et al. (310)	Aerobics Center Longitudinal Study (ACLS), at The Cooper Clinic in Dallas.	To evaluate whether slow HRR after maximal exercise predicts cardiovascular disease (CVD) and all-cause **mortality** among these diabetic men.	n = 2333 men Patients with documented diabetes mellitus (DM) (mean age 49.4 years) diagnosed between 1970 and 1996.	Modified Balke Treadmill Protocol	HR recorded at maximum exercise and at 5 minutes of recovery; Recovery: - Description of recovery protocol not available.	Quartile 1: <55 bpm; Quartile 2: 55 – 66 bpm; Quartile 3: 67 – 75 bpm; Quartile 4: >75 bpm.	Men with DM and slow HRR at 5 min following a maximal exercise test had a higher risk of CVD and all-cause mortality, even after controlling for cardiorespiratory fitness and other confounders.

HRR – heart rate recovery; HR – heart rate; SPEC - thallium201 single photon emission computed tomography; ECG – Electrocardiogram; CAD – coronary artery disease; CVD – cardiovascular disease; DM – diabetes mellitus; CV – cardiovascular; T2DM – type 2 diabetes mellitus; CHD – coronary heart disease.

Table 2.2 – Studies examining the usefulness of HRR as a prognostic marker (continuation).

Reference	Setting	Purpose	Sample	Exercise protocol	HRR protocol	HRR Cutoff values	Main conclusions
Arena et al. (309)	Heart Failure program at the Medical College of Virginia in Richmond.	To assess the prognostic value for **mortality** of HRR, independently and in combination with other cardiopulmonary exercise testing variables, in a group of subjects diagnosed with compensated Heart Failure.	n = 87 (35 female) Consecutive patients diagnosed with heart failure and left ventricle dysfunction.	Modified ramping Treadmill protocol.	HR recorded at maximum exercise and at 1 minute of recovery; Recovery: - no less than 1'30'' recovery; - In treadmill; - 1.0 mph; - 0% incline..	Not presented.	HRR have significant prognostic value for cardiac death and hospitalization in patients with heart failure.
Myers et al. (297)	Veterans Affairs Palo Alto Medical Centers.	To assess the relative prognostic utility of chronotropic incompetence and HRR in predicting cardiovascular **mortality** in patients referred for exercise testing for clinical reasons.	n = 1910 Patients who underwent maximal exercise testing for clinical reasons.	Individualized Treadmill ramp protocol.	HR recorded at maximum exercise and at 1, 2 3 and 5 minutes of recovery; Recovery: - passive recovery; - In supine position.	Low HRR2' < 22bpm	Both chronotropic incompetence and HRR predict cardiovascular mortality in patients referred for exercise testing for clinical reasons.
Chacko et al. (311)	Appropria te Blood Pressure Control in Diabetes (ABCD) trial.	To evaluate the relationship of 1- and 2-min HRR and the incidence of all-cause and CV **mortality**, and CV events in patients with T2DM who had exercise treadmill testing performed on a screening basis.	n = 871 (63% male) Asymptomatic patients with type II diabetes mellitus (T2DM) from ABCD trial evaluated between 1991 and 1993.	Individualized Treadmill ramp protocol.	HR recorded at maximum exercise and at 1 and 2 minutes of recovery; Recovery: - 2' recovery; - In treadmill; - 1.2 mph; - 0% incline..	Low HRR1' < 12bpm	Slowed HRR at 1 and 2 min are useful in assessing risk of mortality and CV events in T2DM patients. Therapies directed at normalizing HRR in this high-risk group of patients should be pursued.

HRR – heart rate recovery; HR – heart rate; SPEC - thallium201 single photon emission computed tomography; ECG – Electrocardiogram; CAD – coronary artery disease; CVD – cardiovascular disease; DM – diabetes mellitus; CV – cardiovascular; T2DM – type 2 diabetes mellitus; CHD – coronary heart disease.

Table 2.2 – Studies examining the usefulness of HRR as a prognostic marker (continuation).

Reference	Setting	Purpose	Sample	Exercise protocol	HRR protocol	HRR Cutoff values	Main conclusions
Carnethon et al. (18)	The Coronary Artery Risk Development in Young Adults (CARDIA) study.	To investigate the association of a comprehensive set of sociodemographic characteristics, health behaviors, and clinical characteristics with the development of slow HRR in healthy young adults who were followed during 20 yr.	n = 2730 (56% female) (age=25,1±3,6 at baseline) Young adults from a healthy cohort followed for 20 years.	Modified Balke Treadmill protocol.	HR recorded at maximum exercise and at 2 minutes of recovery; Recovery: - 2' recovery; - Active walking; - In treadmill.	Low HRR2' < 22bpm	Higher risk for mortality associated to HRR is modulated by the presence and development of CHD risk factors. The characteristics most strongly associated with the odds of having slow HRR in middle age are modifiable.

HRR – heart rate recovery; HR – heart rate; SPEC - thallium201 single photon emission computed tomography; ECG – Electrocardiogram; CAD – coronary artery disease; CVD – cardiovascular disease; DM – diabetes mellitus; CV – cardiovascular; T2DM – type 2 diabetes mellitus; CHD – coronary heart disease.

2.3.3.2. Autonomic nervous system and adipocyte lipid metabolism

This section focus on some aspects of metabolic autonomic control that helps understand the link between ANS and BC, along with some co-morbidities. The ANS is directly involved in regulation of cardiovascular system and secretory glands (including endocrine) and, therefore, can have an important contribution in energy balance. The activation of the SNS is associated to an increase of the metabolic rate, favoring catabolism, which is dominant in stress situations such as exercise or exercise testing, therefore it is often referred to as the 'flight or fight' response therefore can significantly increase energy expenditure (293). On the opposite direction, the PNS affects metabolism favoring anabolism, which is important and mostly dominant in a resting state, therefore it is often referred to as the 'rest and digest' response and so has an important role in energy saving and storing (293). The "Mona Lisa Hypothesis" (an acronym for "Most Obesities kNown Are Low In Sympathetic Activity") as a possible explanation for obesity, was suggested more than two decades ago (313), and still the link and contribution of the ANS on BF content and distribution is under constructive and growing discussion.

Previous reports have already shown ANS imbalance to be linked to obesity (313, 314) and higher BF accumulation (315, 316), diabetes (317-319) and other (320-323), all of which contribute to mortality. On the other way around, BF distribution may also be an important factor, more than whole BF, for the development of ANS imbalance (315, 324, 325).

Adipocyte lipid turnover is an important feature of lipid metabolism which involves adipocyte fatty acids secretion and β oxidation (lipolysis or adipocyte fat catabolism) and fatty acid uptake and *de novo* synthesis (lipogenesis or adipocyte fat anabolism) (326, 327). To stimulate lipolysis of triglycerides within the adipocytes and fatty acids release from adipocytes into the bloodstream, resulting in adipocyte lipid content reduction, a cascade of cellular signals must take place. Lipolysis of adipocyte triglycerides is highly mediated by hormone sensitive lipase (HSL) which, once phosphorylated and activated, moves to the surface of the lipid droplet within the adipocyte cytosol and participates in triglycerides hydrolysis into three fatty acids and one glycerol (328). On the reverse direction, for lipogenesis to take place and increase adipocytes fatty acids uptake, resulting in adipocyte lipid content augmentation, a different cellular signaling cascade must occur. Adipocyte lipogenesis is highly mediated by lipoprotein lipase (LPL) which is synthetized in and secreted by adipocytes, and then migrates to the lumen of adipocytes capillary where, once activated, it can move to the surface of the lipid droplet in the blood to hydrolyze it into NEFA and glycerol (329). Plasma NEFA will then move into adipocytes while the glycerol will flow to the liver to be converted into glycogen (19).

The ANS action is key in adipocyte lipid metabolism regulation, either in the anabolic lipogenesis as in the catabolic lipolysis (326, 327, 330). The ANS can assure adipocyte lipid metabolism regulation by two main mechanisms: (I) the endocrine and (II) the neural input.

2.3.3.2.1. Autonomic nervous system related endocrine regulation of adipocyte lipid metabolism

Both catecholamines (epinephrine and norepinephrine) and insulin are the major plasma hormones responsible for the endocrine regulation of lipolysis and lipogenesis in human adipocytes (326).

Catecholamines are released as hormones by the endocrine glands at the adrenal medulla, and are regulated by SNS activation (292). Catecholamines can both increase adipocyte lipolysis, by stimulating β-adrenergic receptors (also called adrenoceptors), and can decrease adipocyte lipolysis, by stimulating α-adrenoceptors (326). Albeit seaming physiologically contradictory how catecholamines can stimulate both contrary effects in the same adipocyte cell, it has been suggested a reasonable explanation for such apparent paradox. Both α and β-adrenoceptors have different affinities (different levels of tendency to form a chemical bond with a ligand) and desensitization (inability to be activated after being stimulated for long time) onsets to catecholamine stimulation (331). Accordingly adipocyte α-adrenoceptors (particularly α_2) seam to express higher affinity to

catecholamines at low catecholamine concentration, as usually observed at rest. In higher catecholamine concentration situations, such as exercise or exercise testing, α mediated antilipolytic actions seems absent of lipolysis regulation. Catecholamines act also directly on adipocyte β-adrenergic receptors, on the plasma membrane of adipocytes, to activate lipolysis so that adipocyte fatty acids can be mobilized into the blood to be used as energy substrate (326). Three different β-adrenoceptors have been reported present in adipocytes and, as mentioned before, the different β-adrenoceptors have also been shown to have different affinities and desensitization onsets to catecholamines (table 2.3).

Table 2.3 – Adipocyte adrenoceptors affinities and desensitization characteristics, adapted from Lafontan and colleagues (331).

Catecholamines	Adrenoceptors affinities	Adrenoceptors desensitization resistance
Epinephrine	$\alpha_2 > \beta_2 > \beta_1 > \beta_3$	$\alpha_2 = \beta_3 > \beta_2 \geq \beta_1$
Norepinephrine	$\alpha_2 > \beta_1 \geq \beta_2 > \beta_3$	

In summary the receptor with the lowest affinity to catecholamines (β_3) will remain active in response to prolonged catecholamine exposure and, consequently, may be important in prolonged stimulation (such as during prolonged exercise), after the other β-adrenergic receptors become desensitized. Also important is the fact that β-adrenergic receptors have a heterogeneous distribution throughout adipocytes from different body regions and this may be a key feature in regional lipolysis regulation and determinant for BF distribution (326). Because adipocytes from different body regions can have different β adrenoceptors they will respond to stimulation in different ways: visceral adipocytes have β adrenoceptors more easily stimulated but tend do desensitize quite rapidly when being continuously stimulated whereas peripheral/limbs adipocytes have higher expression of β_3 adrenoceptors that are more difficult to activate but can be stimulated during longer periods of time without reducing catecholamine sensitivity (326). The other key hormone in the regulation of lipolysis, insulin, is released by pancreatic β-cells, which are regulated by several inputs, including both SNS and PNS post-ganglionic innervation (19). The SNS activation has an inhibitory effect on insulin secretion while PNS activation will stimulate insulin secretion resulting in increased blood insulin concentration (292). Adipose tissue is very sensitive to changes in insulin concentration. Small increases in plasma Insulin concentration may inhibit lipolysis up to 50% below basal levels by reducing the signaling cascade responsible by activating HSL (326). Besides having an anti-lipolytic effect, Insulin stimulates also LPL activity, which in turn will hydrolyze

chylomicron and very-low density lipoprotein triglycerides, in the blood, increasing blood NEFA concentration (329, 332). Insulin also stimulates NEFA esterification within adipocytes which means that, together with HSL suppression, insulin can significantly reduce NEFA concentration within adipocytes (329). This insulin stimulated NEFA concentration increase in the blood and decrease in the adipocytes will create a concentration gradient favoring NEFA flow from the blood into adipocytes where they are re-esterified (329). As in the muscle cells, insulin also stimulates GLUT4 in adipocytes, increasing glucose uptake (19). Fatty acids entering into the adipocyte will then be converted in triglycerides when associated with one glycerol, while glucose will be either catalyzed into acetil-COA and ultimately converted into fatty acids that will be stored as triglycerides as well (*de novo* lipogenesis) or transformed into α-glycerol phosphate to form triglycerides (because adipocytes are not able to phosphorylate glycerol and therefore has to form glycerol from glucose) (19, 333).

Catecholamines and insulin are recognized as the major plasma hormones regulating lipolysis however other hormones can also influence lipolysis and lipogenesis as well. Growth hormone along with thyroid hormones and cortisol seam to increase the catecholamine stimulated lipolysis (326, 334). Other factors like insulin-like growth factor-1, prostaglandin, adenosine and neuropeptide Y seem to have an inhibitory effect, synergic to insulin, on adipocyte lipolysis (326, 334).

Bottom line the SNS can mainly stimulate lipolysis either by augmenting norepinephrine and epinephrine release at the adrenal medulla or by the inhibition of insulin production at the pancreas. PNS can modulate lipolysis by stimulating insulin secretion to the bloodstream. Lipogenesis is mainly controlled by insulin, and insulin release rate into the blood results from the balance between SNS and PNS activation. The ANS can also modulate adipocyte metabolism by its' neuronal input rather than exclusively by an endocrine pathway as discussed ahead.

2.3.3.2.2. Autonomic nervous system neuronal regulation of adipocyte lipid metabolism

ANS innervation of adipose tissue and its' importance is not yet a consensual topic. Two main discussions concerning ANS innervation of adipose tissue seem presently pinnacling: (I) the importance of the ANS in controlling lipid metabolism in adipocytes, as compared to endocrine regulation (335); (II) the existence of parasympathetic innervation in adipose tissue (335-337). It has been accepted that both catecholamines and insulin are the major regulators of adipocyte lipid metabolism. However this knowledge is being challenged by the possibility that an important participation of the ANS may directly regulate lipid metabolism through its' neural terminations on adipocytes (335). Timothy Bartness is one prominent researcher, among others, who have been

arguing that the participation of ANS neural regulation in the control of lipolysis might be more important than the regulation of lipolysis by adrenal epinephrine (330). Arguments for this rely on experiments that showed, in animal models, that lipid mobilization from adipocytes was almost unaffected in the absence of epinephrine, which occurred after removing its' source (bilateral adrenodemedullation) (338). Thus evidences of direct neuroanatomical projections of post ganglionic neurons from SNS to adipocytes have been presented (339). Furthermore, the presented evidences on SNS innervation of adipose tissue, seams to support the existence of a significant distinction in post-ganglionic neurons, within the SNS, that innervate distinct BF depots (e.g. Inguinal adipocytes *vs* epididymal adipocytes) (339). Such evidences of diverse innervation in different BF depots have been further detailed (337). As a result it has been showed that fatty acids are preferentially recruited from internally located adipocytes, whereas the more externally located subcutaneous adipocytes are relatively spared, in response to different photoperiods (day lengths) which is considered to have a modulation effect on SNS (339). Previous studies had already suggested an effect on BF distribution resulting from the lesion of different regions of the hypothalamus (340). Together these findings suggest a strong possible role of the ANS in modulating BF distribution. Overall these results support a key contribution of the ANS in the neuronal regulation of adipocyte lipid metabolism and also in BF distribution.

The presence of PNS innervation in adipocytes was considered to be absent or irrelevant (330, 341). Nevertheless Kreier and colleagues (336) had shown, and later substantiated (337) and enlightened (342), that PNS input is present in adipose tissue and may have a direct modulatory effect on adipocyte lipid metabolism. Still this is not yet consensual (335). Nevertheless, Kreier and colleagues (336) showed that PNS denervation of adipocytes impressively reduced insulin dependent uptake of circulating glucose and free fatty acids, and increased the activity of HSL, meaning PNS can influence adipocyte lipid metabolism un an anabolic way. Plus, the results by Kreier and colleagues (336) suggest that intra-abdominal and subcutaneous BF depots seem to be innervated by different PNS neurons meaning PNS may have a important role in BF distribution, as was earlier reported for SNS innervation (339). A follow up study confirmed the presence of separated groups of neurons projecting either to the intraabdominal or to the subcutaneous BF depots (337).

The involvement of the ANS in the etiology of obesity and excess BF has long been suggested (the MONA LISA hypothesis) (313). ANS may also be involved in the modulation of BF distribution, as different BF depots were found to be innervated by different selective groups of neurons from both the PNS (337) and the SNS (339), and may, to same extent, be involved in the etiology of adverse BF distribution profiles. The neuroanatomical evidence for a reciprocal influence of BF and ANS, presented by Kreier and colleagues (337), supports a possible novel mechanism for ANS control of fat

metabolism, but also sets a rational for the contribution of BF accumulation, particularly that of the abdominal region, for ANS imbalance. Based on these findings it was hypothesized that ANS imbalance as a consequence of excess BF content, particular that of the intra-abdominal region, may affect autonomic control of biological functions such as cardiac function, glucose metabolism or even liver fat metabolism (337). Some metabolic impairments, particularly IR and obesity (main risk factors for hepatic steatosis), have been shown to precede the presence of slow HRR (18, 343). The distribution of fat throughout the body appears to be particularly important for cardiac autonomic control (315, 324). These evidences arisen the importance, particularly in higher risk subpopulations, of finding clinical non-invasive morphologic and metabolic markers, and potential therapy targets, related to cardiac autonomic control, a known strong independent risk factor for death and other hazardous outcomes.

Chapter 3 – Methods

"Sample, instruments and methods used for the study of body composition and cardiac autonomic control, in the present thesis."

The present chapter provides a comprehensive description of the studied subjects as well as the methodology used for subjects' assessment and data record. The purpose of this is to avoid repetitions of methods descriptions in the subsequent chapters, concerning each conducted study. So, the following chapters will mention the studied variables, referring the present chapter for methods description. Also data analysis description is specific of each individual study within the present research and therefore will only be briefly mentioned in the present chapter and will be presented in detail in each of the following chapters, regarding each study.

This chapter is divided into two main parts. The first part includes "sample" and "study design" subsections and present a description of the sample and how patients were recruited. The Second part focuses on the description of the methodology used for patients' assessment and data record and analysis, including "body composition", "body fat distribution", "cardiac autonomic control" and "statistics" subsections. Even though the full assessment protocol is mentioned in the description of study design, only the methods used to collect data concerning the variables that were studied in the present research were described in detail in the current thesis.

3.1. Sample

To be selected for the present study individuals had to be diagnosed with non-alcoholic fatty liver disease (NAFLD), through liver biopsy or ultrasound, over 18 years of age, without history of hepatotoxic substances intake (eg. steroids) and tobacco consumption. Exclusion criteria included alcohol consumption over 20g/day; the presence of other potential causes for fatty liver disease, including viral hepatitis, auto-immune disease and others; any physical and/or mental disabilities or any condition that constituted an absolute restriction to exercise, or other diagnosed diseases, except for metabolic and cardiovascular diseases (insulin resistance, hypertension or dyslipidemia), with mandatory specific pharmacologic therapy.

Medical doctors from Santa Maria Hospital and Curry Cabral Hospital were contacted to participate in the present research project. Two doctors accepted the challenge. A general outline of the present research project was presented to the patients diagnosed with NAFLD by their medical doctor. After knowing about the research project patients were asked if they would like to be contacted by the researcher to receive more detailed information. A phone call was made to all patients who gave their verbal consent. In every phone call the researcher presented himself, presented the project outline, the main goals, as well as the preparation procedures for the

assessments and asked if patients were able and willing to participate. To those who responded affirmatively two visits to the Exercise and Health Laboratory (LabES) were scheduled. There were 59 consecutive patients enrolled in the present study by their medical doctors, based on initial selection criteria; 37 of the selected patients accepted to participate and 28 were found eligible to enter the study, after exclusion criteria was considered. We studied 28 NAFLD patients (19 males, 51 ± 13 yrs, and 9 females, 47 ± 13 yrs), recruited from the outpatient medical departments in Santa Maria Hospital and Curry Cabral Hospital, in Lisbon (Portugal). Subjects were taking one or more of the following medication: platelet inhibitors, angiotensin-converting enzyme inhibitors, nitrates, statins, ezetimibe, nicotinic acid and biguanides with similar use among male and female patients.

3.2. Study design

This study was conducted at the LabES, from the Interdisciplinary Centre for the Study of Human Performance (CIPER) at the Faculty of Human Kinetics, University of Lisbon (Portugal). It consisted of a crossectional study aiming at investigating associations between variables that can help to understand their usefulness in the clinical setting. Data collection was conducted using equipment from four different laboratories within the CIPER: the body composition (BC) assessment laboratory, the cardiorespiratory assessment laboratory, both part of the LabES, and also the human biology laboratory and the biochemistry laboratory. The present research also benefited from the collaboration of different technicians and professionals in data collection: a chemical analysis technician for venous puncture and saliva collection; an anthropometry technician, certified by the International Society for the Advancement of Anthropometry (ISAK), for BC assessment; a cardiologist for the maximal exercise testing and an exercise physiologist who participated in the load and gas analysis monitoring of the maximal treadmill exercise testing, in the resting metabolic rate assessment, in strength assessment, in the physical activity assessment as well as in the nutritional assessment.

Medical doctors from Santa Maria Hospital and Curry Cabral Hospital were contacted to participate in the present research project. Two doctors accepted the challenge. A general outline of the present research project was presented to the patients diagnosed with NAFLD by their medical doctor. After knowing about the research project patients were asked if they would like to be contacted by the project manager to receive more detailed information. A phone call was made to all patients who gave their verbal consent. In every phone call the project manager presented

himself, presented the project outline, the main goals, the preparation procedures for the assessments and asked if patients were able and willing to participate. To those who responded

Two visits to the LabES were scheduled, to all patients how agreed to participate in this study, with a maximum of two weeks interval between the two visits. On the first day we assessed resting metabolic rate (using gas analysis), BC, including Dual Energy X-ray Absorptiometry (DXA) and anthropometry, strength (using both Biodex and Jamar handgrip), physical activity and nutrition (using questionnaires), by the mentioned order. Subjects were allowed to eat a morning snack after strength assessment. On the second visit patients underwent blood and saliva collection followed by a graded maximal exercise testing on a treadmill. On both days patients arrived at the laboratories at 8h in the morning, after an overnight 12-hour fast. All participants signed an informed consent on the first day, before being included in the present study and undergoing any study procedure. All methods used in the present study comply with ethics and Portuguese laws and were approved by Faculty of Human Kinetics institutional review board for human studies. The present investigation also complies with the principles outlined in the Declaration of Helsinki. A detailed description will be presented only for the methods used in data collection concerning variables included in the studies enclosed in the present thesis.

3.3. Body composition

BC was assessed using both laboratory and field/clinical methods. The selected laboratory method was DXA, which was used to assess whole and regional BC (described in subsection 3.3.1). DXA assessed body fat (BF) compartments were included into ratios to assess BF distribution (described in subsection 3.3.2). The field/clinical methods used in the present study consisted of anthropometric measurements. A detailed description of all anthropometric measurements and assessments, as well as the resulting calculated body indexes, are presented in subsection 3.3.3.

3.3.1. Whole and regional body composition assessment

BC was assessed using both DXA and anthropometry. DXA is an image laboratory method, minimally invasive, used for indirect assessment of BC at the molecular level, in a three compartment model. DXA is validated for the assessment of whole and regional BC, providing precise estimates of three specific body compartments: BF, bone, and lean soft tissue (82, 344, 345). DXA has also been used as a reference method in the validation of other BC assessment methods and techniques (24, 346-348), despite presenting some assumptions and often neglected limitations, particularly in the

assessment of lean soft tissue (82). Unlike fat and mineral, who have fairly stable densities, the density of lean soft tissue may vary as a result of the variation of the percentage of its components, which are mainly water and protein (82). In-depth comprehensive information concerning the use of DXA as a method for the assessment of BC may be found elsewhere (82). Whole body DXA scans (Explorer W, Hologic; Waltham, MA, USA; Fan bean mode) were performed to assess patients' whole and regional BC as well as BF distribution (Figure 3.1). Previously conducted repeated measurements in 18 young adults, to assess the instrument/methods' precision, allowed the calculation of the coefficient of variation (**cov**) for the studied variables obtained from DXA measurements in our Laboratory (Table 3.1).

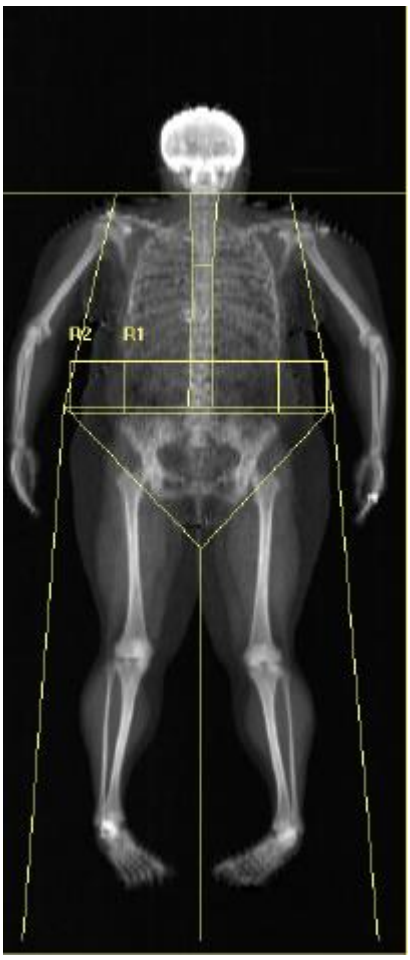

Figure 3.1 – DXA scan image with marked regions of interest. R1 – region of interest 1, as defined by the area within the upper edge of the second lumbar vertebra and de lower edge of the fourth lumbar vertebra.; R2 – region of interest 2 defined as defined as R1 but limited by the rib cage on both sides.

Quality control of DXA equipment and software was made every morning with spine phantom before patients' arrival to the LabES. Also, once a week, every week, quality control included step phantom analysis. All scans were made in the morning after: an overnight 12-hour fast; arriving to the LabES at 8h in the morning; signing an informed consent and undergoing resting metabolic rate assessment (using gas analysis). Before the scan patients were asked to wear light clothes and remove all metallic objects they might have on them, including jewelry, metallic zippers, bras with metal parts, removable prosthesis and other. Patients were positioned on the DXA table, for the whole body DXA scan, in a supine position, and all related procedures, were conducted according to the standard guidelines (349). By default DXA software (QDR for windows, version 12.4) estimates whole and regional BC, including the head, trunk, arms and legs, both left and right, regions fat content, according to a three-compartment model (BF mass, bone mass, and lean soft tissue). The trunk region of interest (ROI) includes chest, abdomen and pelvis. Appendicular ROI includes both arms plus both legs. A list and description of DXA variables is available on table 3.1. All scans analyses were performed, analyzed and submitted to additional analysis by ROI to assess fat content of the abdominal (Abd) and central abdominal (CAbd) regions, by the same observer (Figure 3.1). The abdominal and central abdominal regions were

Table 3.1 – Description and coefficients of variation of variables obtained in 18 young adults, using Dual Energy X-ray Absorptiometry.

DXA variables	Description	COV
Whole BF	body fat of the whole body	0.017
Whole %BF	Percentage of fat in whole body = (total BF/Weight) x 100	0.015
Trunk BF	Sum of body fat from chest, abdominal and pelvis regions	0.005
Trunk %BF	Percentage of fat in the trunk = (trunk BF/trunk mass) x 100	0.005
Appendicular BF	Sum of body fat from both arms and both legs	0.004
Appendicular %BF	Percentage of fat in the appendages = (appendicular BF/appendicular mass) x 100	0.004
Abdominal BF	Includes all fat of the torso comprised between the upper border of L2 and the lower border of L4.	0.010
Abdominal %BF	Percentage of fat in the abdominal region = (abdominal BF/abdominal mass) x 100	0.010
Central Abdominal BF	Includes all fat as in abdominal BF but excluding lateral subcutaneous BF, as delimited by the rib cage.	0.001
Central Abdominal %BF	Percentage of fat in central abdominal region = (central abdominal BF/central abdominal mass) x 100	0.001

DXA – Dual energy x-ray absorptiometry; COV – coefficient of variation; BF – body fat; L2 – second lumbar vertebra; L4 – fourth lumbar vertebra.

determined as we have described elsewhere (350, 351), to access abdominal (Abd) BF and central abdominal (CAbd) BF in accordance to previous works (79, 80). In detail, the upper and lower limits of the abdominal and central abdominal ROI were determined as the upper edge of the second lumbar vertebra to the lower edge of the fourth lumbar vertebra, respectively (79, 80, 350, 351). The sides' limits of the abdominal ROI were determined as to include all trunk length, but exclude any upper limb scan area (79, 350, 351), whereas the vertical sides of central abdominal ROI were the continuation of the lateral sides of the ribs cage, as to exclude the lateral subcutaneous fat of the trunk, including the anterior and posterior subcutaneous abdominal fat, as well as the intra-abdominal fat (80), as can be observed in figure 3.1. The selected ROI assessed by DXA have been shown to be highly correlated to visceral fat, assessed by magnetic resonance imaging (79, 80). All results concerning the absolute and relative BF content variables were registered to the nearest 0.01kg and 0.1%, respectively.

3.3.2. Body fat distribution

BF distribution variables were calculated using ratios between BF content absolute values of different BF depots, obtained by DXA, as we reported before (351). The trunk BF-to-appendicular BF ratio, also called trunk-to-extremity fat ratio (91) or central-to-peripheral fat mass ratio (263), was calculated as the trunk BF content divided by the sum of the BF content of the upper and lower limbs, both left and right. This ratio has been used to assess BF distribution and it intends to identify if BF is more accumulated in the periphery, including both arms and legs, also known as appendages, or if BF is more centrally distributed in the body, particularly in the trunk. This variable has been used even as a criterion marker of BF distribution in order to assess BF distribution prediction capacity of clinical instruments and techniques (263) and have been found among the best correlates to visceral and subcutaneous abdominal fat, in adolescent girls (91). The abdominal BF-to-whole BF was calculated as the selected abdominal ROI BF content divided by the whole BF. This intended to assess how much of whole BF is deposited in the abdominal region. The abdominal BF-to-trunk BF ratio was calculated as the fat content of the selected abdominal ROI divided by the trunk BF. This variable intended to assess the BF distribution within the trunk, in other words, to see the proportion of trunk BF that is centered in the abdominal region. The abdominal BF-to-trunk BF ratio and the abdominal BF-to-Total BF ratio have been used to assess BF distribution changes (351). Ratios were registered to the nearest 0.01. Some of the anthropometric measurements and indexes, included in the present, may also be considered BF distribution surrogates, particularly waist-to-hip ratio (WHR), however, for matters of convenience, all anthropometric variables were presented in the following subsection of the present thesis (see subsection 3.3.3).

3.3.3. Anthropometry and body indexes

As mentioned before, patients also benefited from an anthropometric assessment. Anthropometric measurements included weight, height, skinfolds, lengths and body circumferences. The present thesis includes only body mass, referred here as weight; stature, mentioned here as height and body circumferences, together with resulting body indexes. The studied anthropometric measurements are described in detail in tables 3.2 to 3.8. The choice for such selected anthropometric instruments and techniques was based on the aims and main focus of the present research, which are directed for the clinical practice. So the choice relied on the instruments and assessment techniques that have been suggested to possibly be more easily accepted by professionals for routine practice in clinical settings (23, 102, 166, 167, 169). Body circumferences consisted of arm circumference (arm-C), Hip circumference (hip-C), thigh circumference (thigh-C),

calf circumference (calf-C) and the WC measured according to four different measurement protocols (see tables 3.4 to 3.8). Some standardization procedures were taken into account, as recommended by Agarwal and colleagues (352), latter recommended by the World Health Organization (WHO) (191), to avoid any bias in the measurements, therefore all anthropometric measurements were made with subjects in a standing comfortable position, in their underwear, in over 12-hour fasting state. All measurements were made by the same technician, who was a trained level 2 technician, certified by the International Society for the Advancement of Kinanthropometry (ISAK). All subjects were able to stand without support and were measured in an upright position (168, 170, 353). Body weight was measured to the nearest 0.1 kg, and height was measured to the nearest 0.1 cm, on a digital scale (model 701, Seca; Hamburg, Deutschland) with an attached telescopic stadiometer (model 220, Seca; Hamburg, Germany), The measurement of body circumferences was conducted using an inelastic flexible metallic tape (model W606PM, Lufkin; Vancouver, Canada) and with the help of a segmometer (model segmometer 4, Rosscraft; Canada) to determine some measuring sites. All circumferences measurements were made with the tape parallel to the floor, in the case of both waist and hip-C, or perpendicular to the long axis of the limb, in the case of arm, thigh and calf-C. Body circumference measurements were taken after a tidal expiration, to the nearest 0.1 cm. Height was measured after a deep inhalation.

Table 3.2 – Description of weight measurement

Weight - measurement description (167, 168, 171, 353)
Definition: Measured as body mass assessed in a standard gravitational field, and referred to as weight.
1- Positioning the patient: Patients were standing on the center of the scale, without support and with weight distributed evenly on both feet.
2- Measurement: After the patient was correctly positioned the observer recorded the stable digital readout of the scale.

Table 3.3 – Description of height measurement

Height - measurement description (167, 171, 353)
Definition: Measured as the distance between the highest point of the cranium (vertex) and the horizontal surface where the patients' feet are standing, in an upright position. 1- Positioning the patient: Patients were standing with heels together and toes apart with feet forming a 60^{o} angle, with heels, buttocks and scapulae touching the vertical plane of the stadiometer, and head in the Frankfort plane. 2- Measurement: After the patient was correctly positioned the observer lowered the stadiometer horizontal head piece to the patients head compressing the hair. The observer recorded the result after instructing the patient to stand as tall as possible and to take a deep breath.

Table 3.4 – Description of arm circumference measurement

Arm circumference - measurement description (72, 167, 171, 353)
Definition: Measured as the circumference of the arm, perpendicular to the long axis of the arm. 1- Positioning the patient: Patients were standing in an upright position, with the observer facing his/her right side, and the arms hanging freely at the sides of the trunk with the palms facing the thighs. 2- Measuring site: Measured at the midpoint of the arm with the patient standing erect, with the arms hanging freely at the sides of the trunk with the palms facing the thighs. The observer located the superior lateral tip of the acromion and marked it with a pen on the patients' skin. The observer then located the midpoint of the olecranon process, with the elbow flexed at 90^{o} and measured the distance between both mentioned landmarks with a segmometer. The observer calculated the mid-distance and marker it on the patients' skin with the help of a pen and the segmometer. Midpoint of the arm was located at the mid-distance between the superior lateral tip of the acromion and the midpoint of the olecranon process with the elbow flexed at 90^{o}. 3- Measurement: The observer held the measuring tape case in the left hand and uncoiled the tape so that it passed behind the arm of the subject. The case of the tape changed to the right hand and the tip changed to the left, so that the zero end of the tape was placed below the measurement value, when both ends were crossed at the front of the lateral face of the arm, wrapped around the arm, positioned perpendicular to the long axis of the arm and fitting tight around the arm without compressing the skin. The observer asks the patient to breath normally and records the result attained after a normal exhalation.

Table 3.5 – Description of waist circumference measurement

Waist circumference - measurement description
Definition: Measured as the circumference of the waist in a horizontal plane. 1- Positioning the patient: Patients were standing in an upright position facing the observer, with the arms crossed over his/her chest. 2- Measuring site: Waist circumference (WC) was measured at four distinct sites: **WC1** – Also called minimal waist. Measured at the level of the observed narrowest part of the torso. This is the protocol endorsed by the International Society for the Advancement of Kinanthropometry (ISAK) (168); by the Anthropometric Standardization Reference Manual (170); by the American College of Sports Medicine (ACSM) (72); and others (167). **WC2** – Measured at the level of the uppermost lateral border of the iliac crest. The observer stood on the patients right side to palpate the iliac crest and draw a horizontal line just above the uppermost lateral border of the iliac crest, crossed this horizontal mark with a vertical midaxillary line. This is the protocol endorsed by the National Institute of Health (NIH) and the Centers for Disease Control and Prevention (CDC) of the United States of America, to be used in the National Health and Nutrition Examination Survey (NHANES) (171, 190); and by the Canadian Society of Exercise Physiology (CSEP) (102, 189) **WC3** – Measured at the mid-distance between the lowest rib and the top of the iliac crest. The observer stood on the patients' right side to palpate the iliac crest and draw a horizontal line just above the uppermost lateral border of the iliac crest. Then the observer palpated lowest rib on the right side of the torso. With a segmometer, the observer measured the distance between both marks and calculated the mid distance, to draw a correspondent horizontal line on the right side of the patients' torso. This is the protocol endorsed by the WHO (86, 125, 191) **WC4** – Measured at the level of the umbilicus. This protocol is among the most used protocols and has been used in studies regarding NAFLD (51, 68). 3- Measurement: The observer held the measuring tape case in the left hand and uncoiled the tape so that it passed behind the torso of the subject. The case of the tape changed to the right hand and the tip changed to the left, so that the zero end of the tape was placed below the measurement value, when both ends were crossed at the front of the torso, wrapped around the waist. The tape was positioned over the identified measuring site, on a horizontal plane around the waist, fitting tight without compressing the skin. The observer asks the patient to breath normally and records the result attained after a normal exhalation.

Table 3.6 – Description of hip circumference measurement

Hip circumference - measurement description (72, 167, 168, 353)
Definition: Measured as the gluteal circumference at the level of their greatest posterior protuberance, on a horizontal plane. 1- Positioning the patient: Patients were standing in an upright position with the arms crossed over their chest. The observer faced the patients' right side. 2- Measuring site: Measured horizontally at the level of the observed maximum posterior protuberance of the buttocks. 3- Measurement: The observer held the measuring tape case in the left hand and uncoiled the tape so that it passed behind the torso of the subject. The tape was positioned over the left hip, buttocks and pelvic regions, with the observer standing on the patients' right side. The tape was positioned at the visible maximum posterior protuberance of the buttocks. The tape case changed to the right hand and the tip changed to the left, so that the zero end of the tape was placed below the measurement value, when both ends were crossed over the right hip so that it was wrapped around the hips, on a horizontal plane, fitting tight without compressing the skin. The observer asked the patient to breathe normally and records the result attained after a normal exhalation.

Table 3.7 – Description of thigh circumference measurement

Thigh circumference - measurement description (72, 167, 168, 170)
Definition: Measured as the mid-thigh circumference, perpendicular to the long axis of the thigh. 1- Positioning the patient: Patients were standing in an upright position facing the observer, with feet about 10 cm apart. 2- Measuring site: Measured at the mid distance between the midpoint of the inguinal crease and the proximal border of the patella. Patients sat erect with the knees flexed to about 90^{o}. The observer found both mentioned landmarks and measured the distance between them with a segmometer. The observer then calculated and marked on the patients' thigh the mid-distance between both landmarks. 3- Measurement: The observer holds the measuring tape case in the left hand and uncoils the tape with the right hand so that it passes behind the thigh of the subject. The tape was positioned perpendicular to the long axis of the thigh. The case of the tape changed to the right hand and the tip changed to the left, so that the zero end of the tape was placed below the measurement value, when both ends were crossed over the front of the thigh, with the tape wrapping around the thigh, fitting tight without compressing the skin. The observer asks the patient to breath normally and records the result attained after a normal exhalation.

Table 3.8 – Description of calf circumference measurement

Calf circumference - measurement description (72, 167, 168, 170)
Definition: Measured as the maximum circumference between the knee and the ankle. 4- Positioning the patient: Patients were standing in an upright position facing the observer, with feet about 20 cm apart and weight distributed equally on both feet. 5- Measuring site: Measured the maximum circumference between the knee and the ankle. 6- Measurement: The observer holds the measuring tape case in the left hand and uncoiled the tape with the right hand so that it passed behind the calf of the subject. The tape was positioned perpendicular to the long axis of the leg. The case of the tape changed to the right hand and the tip changed to the left, so that the zero end of the tape was placed below the measurement value, when both ends were crossed over the front of the leg, so that the tape was wrapped around the leg, fitting tight without compressing the skin. The observer asks the patient to breath normally and records the result attained after a normal exhalation.

WC measurement was conducted with four different protocols in each patient (figure 3.2). The selected protocols for WC measurement are the most used protocols found in the literature and endorsed by prominent authors and institutions/organizations (23, 169). Time length of each WC measurement, including all procedures, from brief initial instruction results registering, was recorded in seconds, to the nearest 1 second, with a standard watch chronometer (model RS800, Polar, Oulu, Finland). Body weight was measured to the nearest 0.1 kg, and height was measured to the nearest 0.1 cm, on a scale with an attached stadiometer (model 770, Seca; Hamburg, Germany), according to a standardized protocol (353). All anthropometric measurements were repeated two times and the limits for the difference between the first and

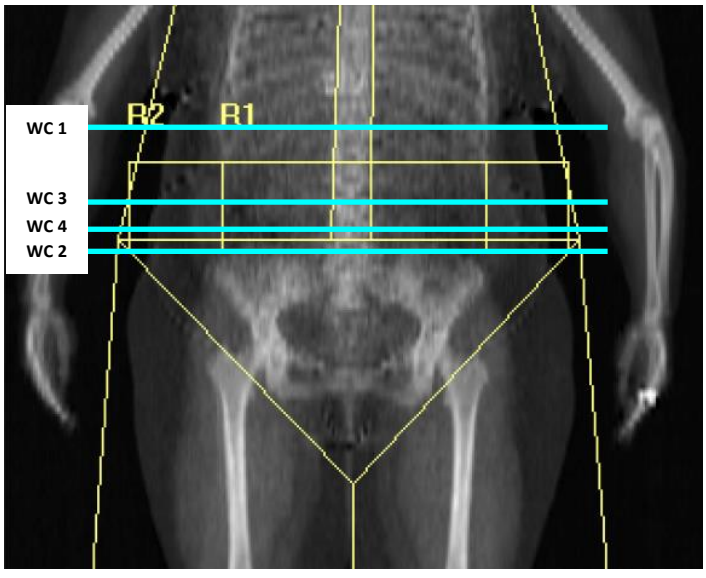

Figure 3.2 – Simulation of waist circumference measuring sites. WC1 – waist circumference measured at minimal waist; WC2 - waist circumference measured just above iliac crest; WC3 – waist circumference measured at mid-distance between lowest rib and iliac crest; WC4 – waist circumference measured at the umbilicus.

second measurements were defined, as presented in table 3.9. Whenever the second measurement differed more than the established limit from the first measurement, a third measurement was carried out. We always considered the result obtained in the second measurement unless a third measurement was carried out. When a third measurement was taken we considered the mode or, if mode was absent, the median value of all three measurements. By using this procedure we sought to always use the most suitable value that was actually measured on the subjects (instead of mean values).

Table 3.9 – Established limits for the difference between first and second anthropometric measurements

Anthropometric variable	Limits for accepted difference between first and second measurements
Weight	0.5 kg
Height	1.0 cm
Arm-C	0.2 cm
WC	1.0 cm
Hip-C	1.0 cm
Thigh-C	0.5 cm
Calf-C	0.2 cm

Arm-C – arm circumference; WC – waist circumference; Hip-C – hip circumference; Thigh-C – thigh circumference; Calf-C – calf circumference.

Several body indexes were calculated, using anthropometric measurements, as surrogates of general BF as well as morphology and fat distribution. Body indexes in the present research included body mass index (BMI), body adiposity index (BAI), WHtR and WHR (table 3.10). Both weight and height were used to calculate the subjects' BMI, by dividing the weight, in kg, by the squared height, in meters (BMI = weight [kg] / height $[m]^2$) (125). The novel BAI, suggested to be a better marker of body adiposity than overall commonly used body indexes, was calculated as hip-C, in cm, divided by height powered to 1.5, in meters, minus eighteen (BAI = (Hip-C [cm] / height $[m]^{1.5}$) - 18) (24); WHtR was calculated as WC divided by height, both in centimeters (WHtR = WC [cm] / height [cm]) (266, 267) and WHR was calculated as WC divided by HC, both in centimeters (WHR = WC [cm] / HC [cm]) (179).

Table 3.10 – Assessed body indexes

Body Indexes	Calculus
BMI	weight [kg] / height [m]^2
BAI	(Hip-C [cm] / height [m] $^{1.5}$) – 18
WHtR	WC [cm] / height [cm]
WHR	WC [cm] / Hip-C [cm]

BMI – body mass index; BAI – body adiposity index; WHtR – waist-to-height ratio; WHR – waist –to-hip ratio; Hip-C – hip-Circumference; WC – waist circumference.

3.4. Cardiac Autonomic Control

Cardiac autonomic control was studied using heart rate recovery (HRR) after a maximal exercise test. Cardiac autonomic control is one feature of the autonomic nervous system (ANS), consequently HRR was the chosen as a marker of ANS function but also, to a lesser extent, as a risk assessment marker. HRR is suggested to be mostly reflective of parasympathetic reactivation, thus providing scarce information about the sympathetic nervous system functioning (296, 304). Slow HRR, however, has been shown to be independently related to higher risk of cardiovascular and all-cause mortality and other cardiovascular and metabolic outcomes (18, 298, 299, 307-311). The next subsections present the description of the exercise protocol as well as the recovery protocol and respective HRR data collection.

3.4.1. Exercise Testing

All subjects underwent a treadmill (model Q-65, Quinton, Cardiac Science Corp; Bothell, WA, USA) graded exercise test (GXT) using Bruce standard protocol (354). All GXT were monitored using a 12 lead electrocardiogram PC-based acquisition module (model PCE-210, Welch-Allyn, Welch Allyn Inc.; Skaneateles Falls, NY, USA) and the data, including heart rate (HR), were monitored and recorded using Welch Allyn CardioPerfect software (Welch Allyn Inc.; Skaneateles Falls, NY, USA). Oxygen uptake was monitored during GXT using a metabolic cart (model CPX Ultima cardio, Medgraphics, Cardio Medical Graphics Corp; St Paul, MN, USA) and data was recorded using Breeze Suite software (version 6.4.1, Medical Graphics Corp; St Paul, MN, USA). Subjects exercised until at least two of the following test termination criteria were reached (355): (1) subjects volitional fatigue; (2) respiratory exchange ratio reached 1.1 or higher; (3) subjects reached predicted maximal HR (HRmax); (4) oxygen uptake did not increase in spite of increasing work load.

3.4.2. Heart rate recovery

Several different protocols have been used to assess HRR, including different exercise protocols and different resting protocols, as can be observed in table 2.2 in the previous chapter. The most commonly used protocol to assess HRR was chosen to be used in the present thesis. When GXT termination criteria were reached patients started exercise recovery with a speed of 1.5 mph (≈2.4 km/h) and incline of 2.5% on the treadmill. Subjects remained walking with the recovery treadmill mechanical load for 2 minutes. After 2 minutes of recovery the treadmill was stopped and subjects continued their recovery seated in an armless standard chair. HR was recorded beat-by-beat and was averaged at 15 seconds intervals for identifying HRmax. HR at the end of the first and second recovery minutes were identified and recorded from beat-by-beat records (HR1 and HR2, respectively). HRR was calculated as the difference between observed HRmax and HR1 (HRR1 = HRmax – HR1) and HR2 (HRR2 = HRmax – HR2). Cut off value for identifying slow HRR was considered 12 bpm for HRR1 (299, 307, 308, 311). The 22 bpm cut off value for identifying slow HRR2 was developed using a supine recovery protocol (298, 312), however it has been used with diverse exercise recovery protocols, including seated (356) and walking (18) recovery protocols and therefore was adopted in the present study for descriptive purposes only.

3.5. Statistics

Considering this is a cross-sectional research, all of the purposes of each study were largely linked to association analysis between variables. For this purpose the statistical analysis consisted mainly of two correlational methods named “partial correlation” and “semipartial correlation”. In order to accomplish a statistical power of 80% ($\beta = 0.20$) at a statistical significance level of 5% ($\alpha = 0.05$), as has been used as a convention (357), a sample of over 84 individuals were needed, therefore 90 patients were coveted to be included in the present sample in the initial research project. This would allow coefficients of correlation as low as 0.3, traditionally corresponding a moderate effect size, to be considered significant and unexposed to type I and II errors (357). Unfortunately, despite all efforts on behalf of everyone involved this research project, only 28 NAFLD patients could be recruited. In order to maintain the statistical power and significance level at the same agreed level, only coefficients of correlation equal or superior to 0.5, corresponding to a large effect size, may be considered unexposed to type I and II errors (357). Other statistical variables and techniques will be used rather consistently, such as coefficient of variance to assess data collection

accuracy, and z statistics for the comparison of different coefficients of correlation. Detailed description of data analysis is further provided in the next chapters, regarding each study.

Chapter 4 – Study 1:

"Is Body Composition and Body Fat Distribution Related to Cardiac Autonomic Control in Non-Alcoholic Fatty Liver Disease Patients?"[1]

[1] Pimenta, N, Santa-Clara, H, Cortez-Pinto, H, Silva-Nunes, J, Rosado, M, Sousa, P, Calé, R, Melo, X, Sardinha, L, Fernhall, B (2013), European Journal of Clinical Nutrition (published online, ahead of print), DOI: 10.1038/ejcn.2013.249.

Abstract

Background: Heart rate recovery (HRR), a cardiac autonomic control marker, has been shown to be a predictor of morbidity and mortality. HRR has been shown also to be related to body composition (BC), yet this was not tested in Non-Alcoholic Fatty Liver Disease (NAFLD) patients.

Purpose: The aim of this study was to determine if, and to what extent, markers of BC and body fat (BF) distribution are related with cardiac autonomic control in NAFLD patients.

Methods: BC was assessed with Dual Energy X-ray Absorptiometry in 28 NAFLD patients (19 males, 51 ± 13 yrs, and 9 females, 47 ± 13 yrs). BF depots ratios were calculated to assess BF distribution. Subjects' HRR was recorded 1 (HRR1) and 2 minutes (HRR2) immediately after a maximum graded exercise test.

Results: BC and BF distribution were related to HRR, particularly weight, trunk BF, central abdominal BF as well as trunk BF-to-appendicular BF ratio showed a negative relation with HRR1 (r=-0.61; r=-0.60; r=0.55 and r=-0.55; respectively, $p<0.01$), and trunk BF-to-appendicular BF ratio was the only also associated with HRR2 (r=-0.59; $p<0.01$, respectively). Age seems to be somewhat related to both HRR1 and HRR2 except when controlled for BF distribution. The preferred model in multiple regression should include trunk BF-to-appendicular BF ratio and BF to predict HRR1 (r2=0.55; $p<0.05$), and trunk BF-to-appendicular BF ratio alone to predict HRR2 ($r^2=0.43$; $p<0.001$).

Conclusions: BC and BF distribution were related to HRR in NAFLD patients. Trunk BF-to-appendicular BF ratio was the best independent predictor of HRR and therefore may be best related to cardiovascular increased risk, and possibly act as a mediator in age related cardiac autonomic control variation.

Keywords: Regional Body Fat; Dual Energy X-ray Absorptiometry; Hepatic Steatosis; Heart Rate Recovery; Parasympathetic Reactivation.

4.1. Introduction

Heart rate recovery (HRR) after exercise is a recognized cardiac autonomic control marker reflective of parasympathetic reactivation (296, 304). Slow HRR is independently related to higher risk of mortality and other cardiovascular and metabolic outcomes (18, 298, 299, 307-311). Autonomic nervous system (ANS) imbalance, including blunted HRR, has also been linked to obesity (313), higher body fat (BF) accumulation (315, 316) and other metabolic disturbances (320, 358). The autonomic nervous system is also known to influence adipocyte metabolism and rather recently Kreier and colleagues (337) presented a neuroanatomical evidence for a reciprocal influence of adipocytes, particularly intra-abdominal, and ANS, and suggested a pathway for ANS mediated imbalance in several other biological functions including liver fat metabolism. Insulin resistance and obesity (main risk factors for hepatic fat accumulation) have been shown to precede the presence of slow HRR (18, 343). Thus, the distribution of BF appears to be particularly important for cardiac autonomic control (315, 324). The relation between BF content and distribution may constitute one missing piece of the puzzle in the link between body composition (BC) and metabolic abnormal outcomes. However this hypothesis needs further research, particularly in specific higher risk subpopulations as are non-alcoholic fatty liver disease (NAFLD) patients. NAFLD is a consequence of imbalanced hepatic fat metabolism and encompasses several stages, from initial hepatic steatosis (hepatocyte fat accumulation), to hepatic inflammation (non-alcoholic steatohepatitis) along with a constellation of other disturbances, that ultimately can lead to advanced fibrosis, cirrhosis, liver failure and death (16). BF distribution, besides being strongly associated with NAFLD is also associated with other metabolic disorders that can also increase the risk of NAFLD, therefore may be an important factor in the etiology of both NAFLD (5, 16) and ANS imbalance (315, 324, 325).

Very few studies have focused on BF distribution and HRR associations and it is unknown if such a relationship exists in NAFLD patients. The purpose of the present study was to determine if, and to what extent, specific markers of BC and BF distribution, are related with reduced parasympathetic reactivation following maximal exercise, as assessed by HRR, in NAFLD patients.

4.2. Methods

In the present study we analyzed two groups of variables as listed in table 4.1: BC variables and ANS variables.

Table 4.1 – List of Studied variables in study 1

Variable	Unit of measurement	Abbreviations	Described in subsection
Age	years	--	3.1
Sex	--	--	3.1
Whole body composition variables			
Weight	kg	--	3.3.3
Stature	cm	--	3.3.3
Body mass index	kg/m^2	BMI	3.3.3
Whole body fat	kg	Whole BF	3.3.1
Whole percentage of body fat	%	Whole %BF	3.3.1
Whole fat free mass	kg	Whole FFM	3.3.1
Whole percentage of Fat free mass	%	Whole %FFM	3.3.1
Regional body composition variables			
Trunk body fat	kg	Trunk BF	3.3.1
Trunk percentage of fat mass	%	Trunk %BF	3.3.1
Trunk fat free mass	kg	Trunk FFM	3.3.1
Trunk percentage of fat free mass	%	Trunk %FFM	3.3.1
Appendicular body fat	kg	Append BF	3.3.1
Appendicular percentage of fat mass	%	Append %BF	3.3.1
Appendicular fat free mass	kg	Append FFM	3.3.1
Appendicular percentage of fat free mass	%	Append %FFM	3.3.1
Abdominal body fat	kg	Abd BF	3.3.1
Abdominal percentage of body fat	%	ABD %BF	3.3.1
Central Abdominal body fat	kg	CAbd BF	3.3.1
Central Abdominal percentage of body fat	%	CAbd %BF	3.3.1
Body fat distribution variables			
Trunk BF-to-Appendicular BF	--	--	3.3.2
Abdominal BF-to-whole BF	--	--	3.3.2
Abdominal BF-to-trunk BF	--	--	3.3.2
Autonomic nervous system variables			
Heart rate recovery at 1’	bpm	HRR 1	3.4
Heart rate recovery at 2’	bpm	HHR 2	3.4

DXA – dual energy x-ray absorptionmetry; bpm – beats per minute; Máx. GXT – maximal graded exercise test; ECG - electrocardiogram.

BC variables, including whole and regional BC, were assessed by DXA, and BF distribution markers were calculated (as described in subsections 3.3.1 and 3.3.2 of this thesis, respectively); and HRR after a maximal exercise test was used as an ANS function marker (soundly detailed in section 3.4 of this thesis), in the studied sample of 28 NAFLD patients (described in section 3.1 of chapter 3).

Descriptive statistics are presented as mean ± sd and range for all analyzed variables. The Gaussian distribution of the data was assessed with the Shapiro-Wilk goodness-of-fit test. Partial and

part, also called semipartial (357), correlations were performed to assess the relations between dependent and independent variables controlling for age and sex. When age was an independent variable the correlation was controlled for sex and fat distribution. Only correlation coefficients equal or above 0.50 were considered to be significant at a significance level of 5% and a statistical power of 80% (357). Multiple linear regressions were conducted, using Enter method, between dependent variables and correlated independent variables to analyze r square change when using two predictors in the model. Stepwise regressions were performed to find preferred models for the prediction of both dependent variables (HRR1 and HRR2). The level of significance was set at $P<0.05$ (two-tailed). Statistical calculations were performed using the IBM SPSS Statistics version 19 (SPSS, inc, Chicago, IL, USA).

4.3. Results

Mean values for all studied variables are presented in Table 4.2. No clinical test interruption criteria, such as electrocardiogram signs of ischemia, new onset of arrhythmias, or excessive hypotensive/hypertensive response, were observed in any GXT. All subjects met termination criteria for ending the GXT. From among the 28 studied NAFLD patients slow HRR1 was present in 6 (22.2%, 2 were female) and slow HRR2 in 5 (18.5%, 2 were female) patients. Neither HRR1 nor HRR2 were different between men and women ($p=0.754$ and $p=0.631$ obtained in an independent samples t test comparison, respectively). Mean body mass index (BMI) of the studied sample was in the overweight category, with no differences between sexes ($p=0.075$ on independent samples t test). BMI was also not related with age ($r= -0.22$; $p=0.285$ on Pearson correlation).

Table 4.2 – Descriptive data of the studied sample in study 1

	NAFLD Patients (n=28)			
Variables	**Mean ± sd ***		**Min. – Max.**	
Age, yr (median, yr)	48.6 ± 12.8	(49)	25 – 68	
Sex, n female (% female)	9	(32.1)		
HRR1, bpm	19.4 ± 10.1		-4.0 – 37.0	
HRR2, bpm	35.9 ± 16.7		-8.0 – 67.0	
Whole Body Analisys				
Weight, kg	88.0 ± 12.8		66.2 – 115.8	
Height, cm	167.3 ± 9.2		149.5 – 183.7	
BMI, kg/m^2 (% obese)	29.1 ± 4.0	(32.1)	22.6 – 42.2	
Whole BF, kg (%)	27.5 ± 9.3	(31.52 ± 8.29)	13.7 – 51.2	(18.84 – 46.28)
Whole FFM, kg (%)	58.8 ± 9.2	(68.48 ± 8.29)	39.6 – 77.7	(53.72 – 81.16)
Regional Body Analisys				
Trunk BF, kg (%)	15.2 ± 5.2	(33.37 ± 7.71)	7.4 – 25.0	(20.87 – 48.01)
Trunk FFM kg (%)	29.9 ± 4.0	(66.63 ± 7.31)	21.1 – 38.6	(51.99 – 79.13)
Append BF, kg (%)	10.8 ± 4.8	(30.42 ± 10.39)	5.2 – 25.7	(13.63 – 50.40)
Append FFM, kg (%)	28.5 ± 5.1	(80.40 ± 6.56)	19.2 – 36.7	(68.64 – 90.66)
Abdominal BF, kg (%)	3.5 ± 1.2	(37.57 ± 6.59)	1.7 – 6.3	(26.09 – 49.40)
Central Abdominal BF, kg (%)	2.9 ± 0.8	(35.82 ± 5.70)	1.6 – 5.0	(24.28 – 44.64)
Body Fat Distribution (Ratios)				
Trunk BF-to-Append BF ratio	1.478 ± 0.371		0.958 – 2.547	
Abdominal BF-to-whole BF ratio	0.130 ± 0.025		0.045 – 0.185	
Abdominal BF-to-Trunk BF ratio	0.231 ± 0.039		0.095 – 0.299	

* results are presented as mean ± standard deviation, unless otherwise noted; BF – body fat; BMI – body mass index; FFM – fat free mass; Append . appendicular; HRR1 – heart rate recovery at 1 min.; HRR2 – heart rate recovery at 2 min.; Máx. – highest observed value; Min. – lowest observed value.

Table 4.3 shows the results for partial and semipartial correlations between each independent variable and each dependent variable (HRR1 and HRR2), controlled for sex and age (unless otherwise noted). Only BF compartments, not fat free mass, were related to HRR. On a whole body analysis only weight was found negatively correlated with HRR1 (p=0.002), in both partial correlations and semipartial correlations. The regional BC analysis showed that trunk BF (p=0.003) and central abdominal (CAbd) BF (p=0.009) were negatively correlated with HRR1 but not with HRR2, both in partial and semipartial correlations, independently of sex and age. The analysis of BF distribution indicated that the trunk BF divided by appendicular BF was the only studied BF distribution marker related to HRR1 (p=0.008) and the only studied independent variable to be related to HRR2 (p=0.003) in both partial and semipartial correlations, when controlled for sex and

age. Age, when controlled for sex and BF distribution, was not related to neither HRR1 nor HRR2 (p=0.596 and p=0.483, respectively).

Table 4.3 – Partial and semipartial correlations between dependent and independent variables of study 1

	HRR 1		HRR 2	
Variables	**r** †	**r** ‡	**r** †	**r** ‡
Age	- 0.12 §	- 0.09 ¶	- 0.154 §	- 0.12 ¶
Whole Body Analisys				
Weight	- 0.61 *	- 0.57 *	- 0.484	- 0.44
Height	- 0.18	- 0.16	- 0.16	- 0.15
BMI	- 0.33	- 0.30	- 0.16	- 0.15
Whole BF	- 0.49	- 0.43	- 0.31	- 0.29
Whole %BF	- 0.24	- 0.22	- 0.07	- 0.06
Whole FFM	- 0.19	- 0.17	- 0.14	- 0.13
Whole %FFM	0.24	0.21	0.19	0.17
Regional Body Analisys				
Trunk BF	- 0.60 *	- 0.55 *	- 0.45	- 0.41
Trunk %BF	- 0.36	- 0.33	- 0.23	- 0.21
Trunk FFM	- 0.21	- 0.19	- 0.15	- 0.14
Trunk %FFM	0.29	0.26	0.26	0.23
Append BF	- 0.27	- 0.25	- 0.10	- 0.09
Append %BF	- 0.02	- 0.02	0.19	0.17
Append FFM	- 0.18	- 0.16	- 0.14	- 0.13
Append %FFM	0.17	0.16	0.14	0.13
Abdominal BF	- 0.49	- 0.45	- 0.27	- 0.24
Abdominal %BF	- 0.30	- 0.27	- 0.09	- 0.09
Central Abdominal BF	- 0.55 *	- 0.51 *	- 0.34	- 0.30
Central Abdominal %BF	- 0.38	- 0.35	- 0.17	- 0.15
Body Fat Distribution (Ratios)				
Trunk BF-to-append BF ratio	- 0.55 *	- 0.50 *	- 0.59 *	- 0.54*
Abdominal BF-to-whole BF ratio	- 0.15	- 0.14	- 0.04	- 0.04
Abdominal BF-to-trunk BF ratio	0.09	- 0.08	0.26	0.24

BMI – body mass index; BF – body fat; %BF – percentage of body baf; FFM – fat free mass; %FFM – percentage of fat free mass; Append – appendicular; HRR1 – heart rate recovery at 1 min.; HRR2 – heart rate recovery at 2 min.; † – partial correlations controlling for age and sex (except when age was an independent variable); ‡ – semipartial correlations removing the effect of age and sex (except when age was an independent variable); § – partial correlation controlling for trunk BF-to-appendicular BF ratio and sex; ¶ – semipartial correlation removing the effect of trunk BF-to-appendicular BF ratio and sex. * - significant for $p<0.05$ and $\beta=0.20$.

All independent variables that showed significant relation with HRR in partial and semipartial correlations were included in multiple linear regression analysis shown in table 4.4. Regressions were performed using only trunk BF-to-appendicular BF ratio and age, which has been suggested to influence HRR in healthy adults (18), as predictors of either HRR1 or HRR2, and also between pairs of independent variables to predict HRR1. Because trunk BF-to-appendicular BF ratio was the only independent variable correlated with both dependent variables, it was chosen as a fixed independent variable in multiple linear regressions. The higher r square change in the prediction of HRR1 seems to be that obtained by adding weight to trunk BF-to-appendicular BF ratio in the prediction model. In the prediction of HRR2 Trunk BF-to-appendicular BF ratio alone was found to predict over 40% of the variation of HRR2, in this sample of NAFLD patients.

Table 4.4 – Linear regressions with R square change analysis (Enter method) between dependent and related independent variables of study 1.

Variables	Model †	R	R square	R square change	P
HRR 1 ‡					
Trunk BF-to-Appendicular BF ratio		0.62	0.38	--	0.001 **
	Weight, kg	0.74	0.55	0.17	0.012 *
	Trunk BF, kg	0.72	0.52	0.14	0.020 *
	C Abd BF, kg	0.66	0.44	0.06	0.138
	Age, yr	0.63	0.39	0.01	0.346
HRR 2 ‡					
Trunk BF-to-Appendicular BF ratio †		0.66	0.43	--	0.000 ***
	Weight, kg	0.71	0.50	0.07	0.087
	Age, yr	0.67	0.44	0.01	0.467

BF – body fat; C Abd BF – central abdominal body fat; HRR1 – heart rate recovery at 1 min.; HRR2 – heart rate recovery at 2 min.; † – Regressions using pairs of independent variables, which include always Trunk BF-to-Appendicular BF ratio plus one of the listed variables; ‡ – Dependent variable in the following regressions. * – significant for p<0.05; ** – significant for p<0.01; *** – significant for p<0.001.

4.4. Discussion

To our knowledge this is the first study to focus on the association between HRR, and BC and/or BF distribution, in NAFLD patients. Most studies on HRR focus primarily on cardiovascular outcomes and have not included BC variables (299, 304, 307-309). Some previous population-based reports showed slower HRR in patients with higher BMI (343, 359). Nilsson and colleagues found

similar results in elders (325). In a recent report, BMI showed the highest odds ratio for slow HRR2 (OR=6.58) over a 20 yr period, after controlling for baseline HRR (18). In our sample, after controlling for age and sex, BMI was not associated with either HRR1 or HRR2. Similar results had also been found in a sample of type 2 diabetes mellitus patients (311). These discrepancies may be explained by differences in studied samples as well as in research protocols, including different HRR record timing criterion as well as considerable exercise protocol differences either in the effort as in the recovery phase. Nevertheless the development of slow HRR seems more likely in those who have more BF accumulation (18, 343, 356).

A recent study reported that the sum of skinfolds accounted for the greatest variance of both HRR1 and HRR2, as compared with BMI, WC and maximal oxygen consumption (316). They used mainly skinfolds from the trunk region, including the abdominal skinfold, which can reinforce the importance of central BC for appropriate ANS function. In accordance to Esco and colleagues the present results showed trunk BF and Central Abdominal (CAbd) BF to be significantly correlated with HRR1, independent of age and sex. Few studies could be found using different BC markers, besides BMI, when focusing on HRR, nevertheless some investigations have used WC to assess central obesity or central as well as whole BF accumulation and found concordant results to ours (18). Mean WC has been shown to be higher in patients with slow HRR (18, 343). The association between slow HRR and WC has been shown to be stronger than with BMI (adjusted for age, race and sex) (343) as well as with all metabolic syndrome components (325). In the present study the results on central BF variables, particularly abdominal fat and central abdominal fat, also show a negative correlation with HRR1, but not with HRR2. Kim and colleagues (315) found somewhat concordant results concerning the relation between visceral fat, particularly that around the myocardium, and both HRR1 and HRR2. The only study we found focusing on HRR and regional BC analysis using DXA showed no differences in HRR between overweight young adults and lean control subjects, in a sample of overnight sleep apnea patients, even though overweight subjects were significantly heavier, and had higher BMI, %BF and central abdominal BF (360). Lindmark and colleagues (358), using heart rate variability (HRV) to assess ANS function, found that increased visceral adipose tissue, but not subcutaneous adipose tissue, as assessed by computed tomography, was strongly associated with ANS imbalance.

In the present study Trunk BF-to-Appendicular BF ratio was the only BF distribution marker that was related to HRR, moreover this BF distribution marker was the only studied independent variable to show correlation magnitudes with both HRR1 and HRR2 that correspond to a large effect

size, even after removing the effect of sex and age. Multiple regression also revealed that other BC variables added little predictive capacity to Trunk BF-to-Appendicular BF ratio. These results emphasize that BF distribution may be more important for ANS function than the absolute or relative amount of BF. Because HRR has been considered a powerful predictor of cardiovascular, as well as overall, mortality (297, 299, 305, 307, 310, 311, 361, 362), the present results suggest that a central BF distribution, particularly Trunk BF-to-Appendicular BF ratio, can possibly relate more strongly to cardiovascular increased risk. The importance of a central distribution of BF was noticed before, using HRV to assess ANS function (324). In that study, abdominal-to-peripheral fat distribution, assess by dividing abdominal by thigh DXA estimated fat contents, was found to explain a significant variation of HRV (324). Carnethon and colleagues (18) showed an association of HRR with aging. In our cross-sectional study the relation of HRR1 and HRR2 with patient's age, was absent if controlled for BF distribution. Christou and colleagues (324) had long suggested that the changes in fat accumulation pattern that occurs with aging, resulting in BF distribution changes, may contribute to the ANS variation attributed to aging. This is a matter that needs to be confirmed either in the general population as in specific subpopulations such as the NAFLD patients and other metabolic impaired subpopulations.

The prevalence of slow HRR in the present study is in accordance with most of the published data, including that from the Cleveland Clinic Foundation (299, 307, 308) that focused on patients referred for symptom-limited exercise testing, as well as in patients with metabolic impairments (310, 311) or in even more heterogeneous populations (343). However, when confronted with healthy cohort data, as shown recently by Carnethon and colleagues (18) the prevalence of slow HRR in the present sample was fairly high. The prevalence of high levels of BMI, including obese and morbidly obese patients, in the present sample was expected since obesity, along with insulin resistance, have been identified as the strongest risk factors for NAFLD, and therefore highly prevalent in this subpopulation (2-4, 6).

In conclusion, in the present study BF content and distribution were important contributors to HRR in NAFLD patients. Excess BF accumulated in the trunk or abdominal regions was associated with poor HRR. BF distribution appears to be more important than overall BF accumulation in explaining the variation of HRR and therefore can possibly be a better predictor of cardiovascular risk in NAFLD patients. The present results may also support an important role of BF distribution in the link between aging and slowing HRR.

Chapter 5 – Study 2:

"Which is the Best Waist Circumference Measurement Protocol to use in Non-Alcoholic Fatty Liver Disease Patients?"[2]

[2] Pimenta, N, Santa-Clara, H, Cortez-Pinto, H, Silva-Nunes, J, Sardinha, L (2013) Nutrition Journal, (Submited).

Abstract

Background: Body Fat (BF), particularly central fat accumulation, is related to Non-alcoholic Fatty Liver Disease (NAFLD). Waist circumference (WC) measurement has been widely used in NAFLD patients as a marker of body composition (BC), particularly central BF, but no single WC measurement protocol (WCmp) is consensually accepted.

Purpose: The aim of the present investigation was to find which of the most used WCmp is preferable to be used in clinical practice with NAFLD patients.

Methods: Total and regional BF were assessed through Dual Energy X-ray Absorptiometry (DXA) in 28 NAFLD patients (19 males, 51 + 13 yrs, and 9 females, 47 + 13 yrs), who were diagnosed by liver biopsy or ultrasound, after exclusion of other potential causes of liver disease. All subjects also underwent anthropometric evaluation including the measurement of WC using four different WCmp (WC1: minimal waist; WC2: iliac crest; WC3: mid-distance between iliac crest and lowest rib; WC4: at the umbilicus).

Results: All WC measurements were correlated particularly with central BF depots, including trunk BF (r=0.78; r=0.82; r=0.82; r=0.84; respectively for WC1, WC2, WC3 and WC4) abdominal BF (r=0.78; r=0.78; r=0.80; r=0.72; respectively for WC1, WC2, WC3 and WC4) and central abdominal BF (r=0.76; r=0.77; r=0.78; r=0.68; respectively for WC1, WC2, WC3 and WC4), even after controlling for age, sex and body mass index. There were no differences between the correlation coefficients obtained between all studied WC measurements and each whole and central analyzed BF variable.

Conclusions: A preferable WCmp in these NAFLD patients could not be clearly established based on biological criteria or on protocols' precision. The WC measured at the superior border of the iliac crest may be a better choice for use in clinical practice based on practical criteria.

Keywords: Regional Body Fat; Dual Energy X-ray Absorptiometry; Hepatic Steatosis; Clinical Body Composition Assessment; Anthropometry.

5.1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a rising recognized condition that has caught a growing attention. In an advanced stage NAFLD can ultimately lead to advanced fibrosis, cirrhosis, liver failure and death (16, 30). In most cases NAFLD is a consequence of an imbalance in hepatic fat metabolism, favouring factors that promote liver fat increase (uptake and synthesis of fatty acids) against factors that promote liver fat reduction (secretion and oxidation of fatty acids) (16, 54). While insulin resistance (IR) seems to be the most consistent and replicable explanation of liver fat accumulation (16, 32), both central body fat (BF) and IR have been found to increase the risk of NAFLD (363), furthermore whole and particularly central BF may also increase the risk for NAFLD by its strong association with IR (5, 183). Excess both whole and central BF accumulation are also known cardiovascular risk factors. These evidences arisen the importance, particularly in this higher risk subpopulation, of finding clinical non-invasive surrogates, and potential therapy targets, of risk related body composition (BC).

Waist circumference (WC) measurement is widely used in different settings and populations (23, 86, 169), including the subpopulation of NAFLD patients (174). WC has been considered a proper surrogate of BC, particularly when focusing on BF distribution (139, 169, 188), and a risk factor for NAFLD (175). WC has also been found to be related with increased morbidity and mortality regardless of WC measuring site (23). In NAFLD patients WC has been found to be associated with several metabolic impairments including IR (183, 184) as well as liver fat (174) and NAFLD severity (185). Moreover, high WC was also found to be related to increased health-care costs (187). Even though widely used, there is currently no optimal and uniquely recommended WC measurement protocol (WCmp) to be used in clinical practice, either in the general population as in specific higher risk subpopulations. Several WCmp have been suggested but scientific rational is lacking to recommend one single protocol (23, 189). Recommended protocols differ mainly on the anatomical landmarks and correspondent measuring sites. The most commonly used WC measurement sites are the midpoint between the lowest rib and iliac crest, the umbilicus and the minimal waist, still a fourth measurement site has also been used and endorsed by the US National Institute of Health (NIH), which is the superior border of the iliac crest (23, 169). Nevertheless several other measuring sites have been sparsely used (23).

To our knowledge this is the first study to look into the usefulness of commonly used WC measurements as surrogates of whole and central BF content in NAFLD patients. Therefore the aim

of the present study was to find which of the most used WCmp is the best for use in clinical settings with NAFLD patients, considering a threefold criteria: the WC most closely associated with whole and central BF content in NAFLD patients; the most precise WCmp; the most practical WCmp to use in clinical practice.

5.2. Methods

In the present study we analyzed two groups of variables (Table 5.1): BC variables, including whole and regional BF content, as assessed by DXA (as described in section 3.3.1); and WC measurements using different measurement protocols (as detailed in subsection 3.3.3), in the studied sample of 28 NAFLD patients (described in section 3.1).

Table 5.1 – List of Studied variables in study 2

Variable	Unit of measurement	Abbreviations	Described in subsection
Age	Years	--	3.1
Sex	--	--	3.1
Anthropometry			
Weight	Kg	--	3.3.3
Height	Cm	--	3.3.3
Body mass index	kg/m^2	BMI	3.3.3
Waist Circumference 1	Cm	WC1	3.3.3
Waist Circumference 2	Cm	WC2	3.3.3
Waist Circumference 3	Cm	WC3	3.3.3
Waist Circumference 4	Cm	WC4	3.3.3
Whole and Regional Body Composition			
Whole body fat	Kg	Whole BF	3.3.1
Whole percentage of body fat	%	Whole %BF	3.3.1
Trunk body fat	Kg	Trunk BF	3.3.1
Trunk percentage of fat mass	%	Trunk %BF	3.3.1
Appendicular body fat	Kg	Append BF	3.3.1
Appendicular percentage of fat mass	%	Append %BF	3.3.1
Abdominal body fat	Kg	Abd BF	3.3.1
Abdominal percentage of body fat	%	ABD %BF	3.3.1
Central Abdominal body fat	Kg	CAbd BF	3.3.1
Central Abdominal percentage of body fat	%	CAbd %BF	3.3.1

DXA – dual energy x-ray absorptionmetry; bpm – beats per minute; Max. GXT – maximal graded exercise test; ECG - electrocardiogram.

Descriptive statistics are presented as mean ± sd and range for all analyzed variables. The Gaussian distribution of the data was assessed with the Shapiro-Wilk goodness-of-fit test. Levenne

test was used for assessing sample variance homogeneity. Paired-samples T test was used to compare WC results obtained with different WCmp. When homogeneity of variance was not present, the corrected significance values of T test were used. The association between whole and central BF and the results obtained with each WCmp was assessed using partial as well as part, also called semipartial (357), correlations, controlling for age and sex and body mass index (BMI). Only correlation coefficients equal or above 0.50 were considered to be significant at a significance level of 5% and a statistical power of 80%. Pairs of correlation coefficients obtained between each WC with each dependent variable were compared, using Z statistic, to find if any WC was more closely associated with whole and central BF. To evaluate the precision of the studied WCmp we calculated coefficients of variation for the repeated measurements, and compared them between WCmp using paired-samples T test. To respond to the third criteria, comprised in the aim of the present study, paired-samples T test was used to compare the time consumption of WC measurement between different measurement protocols. Statistical calculations were performed using the IBM SPSS Statistics version 19 (SPSS, inc, Chicago, IL, USA), except for z statistic which was performed using Medcalc version 11.1.1.0 (MedCalc Software, Mariakerke, Belgium).

5.3. Results

Mean values for all studied variables are presented in table 5.2. From among the 28 studied NAFLD patients obesity was present in 9 subjects (3 were female), according to BMI classification, yet mean BMI showed no differences between sexes (p=0.075 on independent samples t test) and was considered to be in the overweight category for the whole studied sample. BMI was also not related with subjects' age (r= -0.22; p=0.266 on Pearson correlation). Results for WC measurements were considered to be different between all studied WCmp, for instance WC4 showed the highest values whereas WC1 showed the lowest, and WC3 was smaller than WC2, as shown in table 5.3.

Table 5.2 – Descriptive data of the studied sample in study 2.

	NAFLD Patients (n=28)			
Variables	**Mean ± sd ***		**Min. – Max.**	
Age, yr (median, yr)	49.5 ± 12.8	(49)	25 – 68	
Sex, n female (% female)	9	(32,1)		
Anthropometry				
Weight, kg	87.6 ± 12.7		66.2 – 115.8	
Height, cm	167.2 ± 9.2		149.5 – 183.7	
BMI, kg/m^2 (% obese)	29.1 ± 4.0	(32.1)	22.6 – 42.2	
WC 1, cm	100.7 ± 8.2		86.0 – 119.8	
WC 2, cm	104.8 ± 10.6		85.3 – 128.7	
WC 3, cm	103.7 ± 10.4		85.7 – 129.3	
WC 4, cm	106.3 ± 11.5		86.7 – 129.1	
Whole and Regional Body Composition				
BF, kg (%)	27.2 ± 9.3	(31.31 ± 8.20)	13.7 – 51.2	(18.84 – 46.28)
FFM, kg (%)	58.7 ± 9.1	(68.69 ± 8.20)	39.6 – 77.7	(53.72 – 81.16)
Trunk BF, kg (%)	15.2 ± 5.2	(33.15 ± 7.65)	7.4 – 25.0	(20.87 – 48.01)
Trunk FFM kg (%)	29.9 ± 3.9	(66.85 ± 7.65)	21.1 – 38.6	(51.99 –79.13)
Append BF, kg (%)	10.8 ± 4.8	(30.42 ± 10.39)	5.2 – 25.7	(13.63 – 50.40)
Append FFM, kg (%)	24.5 ± 5.1	(69.58 ± 10.39)	14.9 – 34.8	(49.60 – 86.37)
Abdominal BF, kg (%)	3.5 ± 1.2	(37.57 ± 6.59)	1.7 – 6.3	(26.09 – 49.40)
Central Abdominal BF, kg (%)	2.9 ± 0.8	(35.82 ± 5.70)	1.6 – 5.0	(24.28 – 44.64)

BMI – body mass index; WC1 – Waist circumference as measured at minimal waist; WC2 - Waist circumference as measured just above iliac crest; WC3 - Waist circumference as measured at the mid-distance between lowest rib and iliac crest; WC4 - Waist circumference as measured at the umbilicus; BF – body fat; FFM – fat free mass; * Results are presented as mean ± standard deviation, unless otherwise noted; Min. – lowest observed value; Max. – highest observed value; BMI – body mass index; BF – body fat; FFM – fat free mass.

Table 5.3 – Mean differences and P values from paired samples T test used in the comparison between waist circumference results obtained with different measurement protocols.

Variables	WC 1, cm		WC 2, cm		WC 3, cm	
	Dif †	P	Dif †	P	Dif †	p
WC2	4.1	0.000	--	--	--	--
WC3	3.0	0.000	-1.1	0.000	--	--
WC4	5.6	0.000	1.5	0.012	2.6	0.001

WC1 – Waist circumference measured minimal waist; WC2 - Waist circumference measured just above iliac crest; WC3 - Waist circumference measured at mid-distance; WC4 - Waist circumference measured at the umbilicus; † - results from the WC protocols in the Left column subtracted by the results from the WC protocols in the top line.

Table 5.4 shows the results for partial and semipartial correlations between each WC and each whole or central studied BF depot controlled for sex, age and BMI. All WC results were somewhat correlated with the overall studied DXA assessed BF depots, controlling for age, sex and BMI, often showing correlation coefficients above 0.5. Yet, only WC4 was correlated with both absolute and relative whole BF, in partial correlation. WC1, WC2 and WC3 were correlated only with the absolute values of whole BF, in partial correlations. All of the coefficients of correlation found in semipartial correlations for whole BF could not be considered significant. All studied WC were correlated with absolute and relative trunk BF, in partial correlations, however, in semipartial correlations, all WC only correlated significantly with absolute values of trunk BF. WC 1, WC2 and WC3 were associated with absolute and relative values of abdominal fat depots, comprising abdominal (Abd), Abd %BF, central abdominal (CAbd) BF and CAbd %BF, but WC4 was only correlated with Abd BF and Cabd BF, in partial correlations. Semipartial correlations only confirmed the correlation with Abd BF and CAbd BF, for all WC. Table 5.5 shows the results for the comparison between the correlation coefficients listed in table 5.4. Comparisons were made between pairs of competing WC correlations results with each dependent variable. No differences were found between all performed correlations with each studied BF depot. Nevertheless semipartial correlations, when the effect of age sex and BMI was removed, seem to show more constant correlation coefficients across different WCmp, for the same BF depot, than partial correlations, when results are only adjusted for age, sex and BMI. However, as stated, no differences were found across WC measuring sites, in the association with whole and central BF depots.

Table 5.4 – Partial and semipartial correlations between all studied circumferences and body composition variables of study 2.

	WC 1, cm		WC 2, cm		WC 3, cm		WC 4, cm	
Variables	r †	r ‡	r †	r ‡	r †	r ‡	r †	r ‡
Whole BF	0.63*	0.34	0.68*	0.37	0.66*	0.36	0.77*	0.35
Whole %BF	0.46	0.23	0.45	0.22	0.46	0.22	0.54*	0.28
Trunk BF	0.78*	0.52*	0.82*	0.54*	0.82*	0.54*	0.84*	0.51*
Trunk %BF	0.56*	0.35	0.54*	0.34	0.56*	0.34	0.57*	0.30
Abd BF	0.78*	0.68*	0.78*	0.69*	0.80*	0.70*	0.72*	0.66*
Abd %BF	0.59*	0.49	0.57*	0.47	0.58*	0.48	0.47	0.44
C Abd BF	0.76*	0.73*	0.77*	0.74*	0.78*	0.75*	0.68**	0.70*
C Abd %BF	0.55*	0.48	0.53*	0.46	0.54*	0.47	0.48	0.44

WC1 – Waist circumference measured minimal waist; WC2 - Waist circumference measured just above iliac crest; WC3 - Waist circumference measured at mid-distance; WC4 - Waist circumference measured at the umbilicus; BF – body fat; Abd BF – abdominal body fat; CAbd BF – Central abdominal body fat; † - partial correlations between studied circumferences and dependent variables, controlled for age, sex and body mass index; ‡ - semipartial correlations between studied circumferences and dependent variables, removing the effect of age, sex and body mass index; * - significant for $p<0,05$ and $\beta=0.20$.

Table 5.6 shows both the coefficients of variation for the WC measurements according to the studied protocols, as well as the mean ± sd time spent for WC measurements using each of the studied WCmp. Coefficient of variation was not different irrespectively of the WCmp used. Time spent in each measurement was longer for WC3 as compared to all others. WC2 was more time consuming than both WC1 and WC4. The latest showed no differences between both mean time lengths of measurements. In summary time length of measurement of the studied protocols was as follows: WC1 ≈ WC4; WC1 and WC4 < WC 2 < WC3.

Table 5.5 – Z statistic P values for the comparison between correlation coefficients obtained in partial and semipartial correlations between all studied waist circumferences and the dependent variables, as expressed in absolute (lower left half of table) and relative (upper right half of the table) values.

		WC1		WC2		WC3		WC4			
		p†	p‡	p†	p‡	p†	p‡	p†	p‡		
				0.954	0.982	0.971	0.988	0.874	0.841	Whole %BF	WC1
				0.923	0.962	0.980	0.990	0.715	0.853	Trunk %BF	
				0.886	0.920	0.949	0.963	0.747	0.836	Abd %BF	
				0.909	0.928	0.952	0.964	0.836	0.865	C Abd %BF	
WC2	Whole BF	0.744	0.907			0.982	0.994	0.920	0.824	Whole %BF	WC2
	Trunk BF	0.754	0.918			0.944	0.971	0.788	0.828	Trunk %BF	
	Abd BF	0.950	0.968			0.937	0.957	0.899	0.915	Abd %BF	
	C Abd BF	0.912	0.921			0.957	0.964	0.927	0.936	C Abd %BF	
WC3	Whole BF	0.828	0.939	0.913	0.968			0.902	0.830	Whole %BF	WC3
	Trunk BF	0.746	0.918	0.992	1.000			0.734	0.862	Trunk %BF	
	Abd BF	0.799	0.876	0.848	0.908			0.837	0.872	Abd %BF	
	C Abd BF	0.842	0.864	0.929	0.943			0.884	0.901	C Abd %BF	
WC4	Whole BF	0.881	0.958	0.859	0.948	0.946	0.981				
	Trunk BF	0.881	0.958	0.643	0.876	0.635	0.876				
	Abd BF	0.819	0.879	0.770	0.985	0.629	0.758				
	C Abd BF	0.849	0.862	0.763	0.784	0.697	0.730				

WC1 – Waist circumference measured minimal waist; WC2 - Waist circumference measured just above iliac crest; WC3 - Waist circumference measured at mid-distance; WC4 - Waist circumference measured at the umbilicus; † - comparison between correlation coefficients obtained in partial correlations between waist circumferences and all dependent variables, controlled for age, sex and BMI; ‡ - comparison between correlation coefficients obtained in semipartial correlations between waist circumferences and all dependent variables, removing the effect of age, sex and BMI.

Table 5.6 – Coefficient of variation and time length for measurement of each waist circumference protocol used in study 2.

	WC 1	WC 2	WC 3	WC 4
COV, %	0.45	0.49	0.47	0.73
TLM, sec.	35±6*	44±4**	74±4**	34±5***

COV – mean coefficient of variation, TLM – mean±standard deviation time length of measurements, in seconds; WC1 – Waist circumference measured minimal waist; WC2 - Waist circumference measured just above iliac crest; WC3 - Waist circumference measured at mid-distance; WC4 - Waist circumference measured at the umbilicus; * - different from WC2 and WC3 (p<0-001) but not WC4 (P=0.522); ** - different from all other WC TLM (p<0.001); *** - different from WC2 and WC3 (p<0-001) but not WC1 (P=0.522).

5.4. Discussion

Even though WC has been a widely used measure in the subpopulation of NAFLD patients (174, 364), to our knowledge this is the first study to focus on the comparison of the usefulness of different WCmp based on a scientific and practical rational. The prevalence of high levels of BMI, including obese and morbidly obese patients, in the present sample was expected since obesity, along with insulin resistance, have been identified among the strongest risk factors for NAFLD, and therefore highly prevalent in this subpopulation (2-4, 6). WC results were also quite high which was consistent with sample levels of BMI. The magnitude of WC mean values was different according to the protocol in use meaning they are not interchangeable. This has large implications in all sorts of comparisons meaning that only one protocol should be used to permit within subjects comparison (pre - post), between groups comparisons and, most important, subjects results confrontation with normative charts or cut-off values because a dichotomous analysis may amplify even small absolute differences (365). To avoid misclassifications measurements should be made using the same protocol that was used to build the reference values. Several previous studies also reported WC magnitudes influenced by WCmp (365-367). However small or absent differences, particularly in men, between measurements of WC according to NIH (at the superior border of the iliac crest) and to WHO (at the midpoint between lowest rib and iliac crest) protocols have also been reported (365-367). Consequently, it has been suggested that current WC thresholds, generalized using WHO protocol, could be applied to NIH measurements (23). Even though the analysis of absolute values and classification of WC was not the aim of the present study our data do not support this generalization for NAFLD patients, but more research is needed to look into sex differences and other possible influencing variables that may help to endorse or reject an interchangeable use of both NIH and WHO WCmp in NAFLD patients. Moreover fairly recent studies reported differences in the prevalence of metabolic disorders as well as in the prevalence of metabolic syndrome, when comparing the same subjects grouped by results obtained with different WCmp using metabolic syndrome WC cutoff values (368, 369). These results reinforce the necessity for finding one single consensual standardized WCmp.

In the present sample of NAFLD patients, as expected, WC results were highly associated with whole and central BF, adjusted for age, sex and BMI. Correlation coefficient magnitudes often revealed a large effect size ($r>0.5$). The association of WC with BC, particularly with central BF has long been reported (188, 370, 371). Equations using only age and WC, as measured midpoint

between lowest rib and iliac crest, were found to be among the best BC prediction equations (370). WC as measured midpoint between lower ribs and iliac crest was found highly related with intra-abdominal fat, adjusted for age (371). In the present report, the results obtained in partial correlation may suggest that WC1, WC2 and WC3 are better associated with central BF, because they were related to both absolute and relative values of central BF depots, as opposed to WC4 which was the only related with both absolute and relative whole BF. However, when the results obtained in semipartial correlations are taken into consideration it is possible to notice that all coefficients of correlation were fairly reduced, as compared to those obtained in partial correlations. This means that the control variables, age, sex and BMI explained a considerable part of the variation of the studied DXA assessed BF depots, which is not explained by any of the tested WC. As a result, in semipartial correlations, all studied WC were similarly only correlated with trunk BF, Abd BF and CAbd BF, controlled for age, sex and BMI. Wang and colleagues (366) had already found stronger associations between WC and absolute central BF, as opposed to either whole BF mass or any percentage expressed BF depot, assessed by DXA. In the present study, WC appears to be a good BF surrogate, particularly for central BF which is the most hazardous BF, and may be an adequate procedure to assess BF accumulation in NAFLD patients for use in clinical practice, even after removing the effect of age, sex and BMI.

Comparisons between pairs of competing WC correlations results with each dependent variable showed no differences meaning all WC results are similarly associated with the analysed BF depots, irrespectively of the WCmp used. Semipartial correlations, when the effect of age sex and BMI was removed, seem to show more constant correlation coefficients than partial correlations across different WCmp. This may indicate that the variation that may be found in the association between WC measured at different site and BC may be accounted by factors like subjects' age, sex or BMI, other than the site of measurement alone. Nevertheless, in the present results, no differences were found between correlation coefficients across different WC measuring sites. Conflicting results can be found in general population (366, 367). A recent report focused on NIH (at the superior border of the iliac crest) and WHO (midpoint between lowest rib and iliac crest) recommended WCmp, as well as a third WC measurement that was not used in the present study (bellow the lowest rib), and found differences between correlation coefficients in partial correlations with abdominal adipose tissue assessed with magnetic resonance imaging, in women but not in men (367). Wang and colleagues (366) found correlation with both whole and trunk BF, though stronger with trunk BF, regardless of sex and WCmp (bellow lowest rib, at the superior border of the iliac crest, midpoint and

narrowest waist). In a sound review, by Ross and colleagues (23), it was concluded that the use of different WCmp do not change the well-established relationships between WC and morbidity of cardiovascular disease and diabetes and with cardiovascular and all-cause mortality. The present results are in agreement with the lack of biological rational described in previous reports, substantiating the difficulty in finding one single consensual standardized WCmp. Nevertheless there seems to be a relative consensus regarding the need to find a single consensual standardized WCmp.

In the present sample of NAFLD patients all WC measurements, irrespective of WCmp, were similarly correlated with the studied BF depots, even after removing the effect of sex, age and BMI. The present findings confirm, in NAFLD patients, the findings reported on other populations regarding specific WCmp selection for the association with whole and central BF (366, 367). Despite conflicting results found in the literature (368) the differences in WCmp do not seem to influence the relationship between WC measurements and BC associated risk for cardiovascular and other hazardous outcomes (23, 369). In the absence of biological support identifying one preferable WCmp additional criteria have been suggested and may be used to substantiate the decision to use one particular WCmp: the use of bony landmarks and ease of measurement (23, 169, 189). It was argued that the use of bony landmarks could be preferable because of increased precision (169) or reliability (23, 189). WHO WCmp could also be expected to be less reliable than NIH protocol considering it is more exposed to error propagation effect caused by the identification of an additional landmark, measurement of the distance and calculation of the midpoint between the two. The present data do not confirm better precision of one single studied WCmp over another, as assessed by the comparison of coefficients of variation. Present results are in accordance to recent reported data, using exactly the same WCmps used in the present study (365). Other study analyzed measurement reproducibility and found no differences between studied WCmps (366). While the preference for bony landmarks when selecting a WCmp can be understandable because it appears to be less prone to variations or bias, particularly when monitoring changes in weight loss settings, more research is needed to support this argument. The second suggested criteria, ease of measurement, means that WCmp should be easily adopted by general public and practitioners to be elected and should also require less specific training and be less time consuming to be suitable for routine clinical practice (169). It has been suggested that WC measured at the superior border of the iliac crest would be more likely adopted by general public and practitioners as it requires only the palpation of one bony landmark whereas the protocol suggested by WHO (midpoint between lowest rib and iliac crest) requires the detection of two landmarks as well as the calculation of the midpoint between the two,

consequently NIH WCmp would also require less training (189). However published data show similar use among the most common WCmps (22 to 33% minimal waist, 26 to 29% midpoint and 27 to 28% umbilicus) (23, 169) and the identification of bony landmarks seem to require more training and experience for observers to locate then consistently, as subjectively reported by Mason and colleagues (365). Difficulties were also reported for measuring WC using external landmarks (minimal waist and umbilicus) (365). In the present study only the difficulties reported for WC using external landmarks were felt, with the exception of those found for quite thin subjects, as there were not any in the present sample, still lowest rib was the most demanding of bony landmarks to locate. Limited time availability has been suggested as one of the reasons for not using WC measurement in routine clinical practice (169). Manson and colleagues (365) also subjectively reported that WC measurement at the midpoint between lowest rib and iliac crest was more time consuming than when using other studied WCmp, although no quantitative data was shown. The present data confirm the findings that WHO WCmp is the most time consuming of all studied protocols (365). Also WCmp using external landmarks were less time consuming then those using bony landmarks.

The present study confirms the strong association between WC and BF, especially central BF, even after controlling for the effect of age, sex and BMI. However, a preferable WCmp could not be established for the present sample of NAFLD patients based on biological criteria. There seem to be also no precision differences between different WCmp. Practical criteria may somewhat support a preferential use of WC measured at the superior border of the iliac crest, but additional research will be useful to confirm this conclusion regarding the association with other metabolic characteristics and outcomes in NAFLD patients.

Chapter 6 – Study 3:

"Does Waist Circumference Measurement Protocol Influence the Relation Between Waist-to-Height Ratio and Body Composition in Non-Alcoholic Fatty Liver Disease Patients?"[3]

[3] Pimenta, N, Santa-Clara, H, Cortez-Pinto, H, Silva-Nunes, J, Sardinha, L (2014) Clinical Nutrition (submitted).

Abstract

Background: Body Fat (BF), particularly central fat accumulation, is related to Non-alcoholic Fatty Liver Disease (NAFLD), and is related also to increased cardiovascular morbidity and mortality, which is also increased in NAFLD patients. Waist-to-height ratio (WHtR) has been suggested to be the best clinical body index to predict cardiovascular risk. No single waist circumference measurement protocol (WCmp) is consensually accepted nor its effects on WHtR correlation have been assessed.

Purpose: The aim of this study was to analyze the effect of using different WCmp on the strength of association between WHtR and both, whole and central BF in NAFLD patients.

Methods: Whole and regional BF content were assessed with Dual Energy X-ray Absorptiometry (DXA) in 28 NAFLD patients (19 males, 51 ± 13 yrs, and 9 females, 47 ± 13 yrs) , who were diagnosed by liver biopsy or ultrasound, after exclusion of other potential causes of liver disease. All subjects also underwent anthropometric evaluation including the measurement of WC using four different WCmp (WC1: minimal waist; WC2: iliac crest; WC3: mid-distance between iliac crest and lowest rib; WC4: at the umbilicus) and WHtR was calculated using each of the WC measurements (WHtR1, WHtR2, WHtR3 and WHtR4, respectively).

Results: All WHtR measurements were correlated particularly with central BF depots, including abdominal BF (r=0.80; r=0.84; r=0.84; r=0.78; respectively for WHtR1, WHtR2, WHtR3 and WHtR4) and central abdominal BF (r=0.72; r=0.77; r=0.76; r=0.71; respectively for WHtR1, WHtR2, WHtR3 and WHtR4), after controlling for age, sex and body mass index. There were no differences between the correlation coefficients obtained between all studied WHtR and each whole and central analyzed BF variable.

Conclusions: The choice for a particular WCmp does not change the strength of relation between both, whole and central, BF depots in the present sample of NAFLD patients. This study may endorse an interchangeable use of different WCmp to identify subjects above suggested healthy boundary and therefore at higher risk.

Keywords: Regional Body Fat; Dual Energy X-ray Absorptiometry; Hepatic Steatosis; Clinical Body Composition Assessment; Anthropometry.

6.1. Introduction

Waist-to-height ratio (WHtR) is an index of abdominal obesity initially suggested by Hsieh and Yoshinaga in the mid-nineties (266, 267). By then WHtR was suggested to be a better predictor of multiple coronary heart disease risk factors than other obesity and body fat (BF) distribution indexes in both men (267) and women (266). WHtR was further suggested to be preferable to other indexes and clinical assessments, including body mass index (BMI), waist circumference (WC) and waist-to-hip ratio (WHR), to predict cardiovascular risk factors, in different ethnic and age groups (129, 270). WHtR seems also to be at least similarly associated to abdominal fat as is WC, and better than both BMI and WHR (278, 279). To our knowledge only one study focused on non-alcoholic fatty liver disease (NAFLD) patients using WHtR (252). This study found WHtR to be higher in NAFLD patients but was not a better prognostic factor of NAFLD than were BMI or WHR.

There is still some inconsistency considering the WC measurement protocol (WCmp) used to calculate WHtR (280). Several WCmp have been suggested but scientific rational is lacking to recommend one single protocol (23, 169, 189). The association of WC to cardiometabolic risk is independent of WCmp, however measurements using different WCmp have different magnitudes and therefore are not interchangeable (23). Suggested protocols differ mainly on the anatomical landmarks and correspondent measuring sites. WHtR was initially suggested using WC measured at the umbilicus (266, 267). In a fairly recent review on WHtR, WC measured midpoint between the lowest rib and iliac crest was found to be used in 50% of the reviewed papers, and for that reason its routine use was encouraged (280).

To our knowledge it is unknown if the use of different commonly used WC, measured according to different WCmp, which differ mainly in the measuring sites, affects the relation between WHtR and both whole and central BF content. Therefore the aim of the present study was to analyze which of the most used WCmp is better to calculate WHtR for use in clinical practice with NAFLD patients as a surrogate for whole and central BF.

6.2. Methods

In the present study we analyzed two groups of variables (Table 6.1): whole and regional BF content, as assessed by DXA (as described in subsections 3.3.1); and WHtR as calculated using four

different WC measured according to different WCmp for each patient (as detailed in subsection 3.3.3), in the studied sample of 28 NAFLD patients (described in section 3.1).

Table 6.1 – List of Studied variables in Study 3

Variable	Unit of measurement	Abbreviations	Described in subsection
Age	Years	--	3.1
Sex	--	--	3.1
Anthropometry			
Weight	kg	--	3.3.3
Height	cm	--	3.3.3
Body mass index	kg/m^2	BMI	3.3.3
Waist Circumference 1	cm	WC1	3.3.3
Waist Circumference 2	cm	WC2	3.3.3
Waist Circumference 3	cm	WC3	3.3.3
Waist Circumference 4	cm	WC4	3.3.3
Waist-to-height ratio 1	--	WHtR1	3.3.3
Waist-to-height ratio 2	--	WHtR2	3.3.3
Waist-to-height ratio 3	--	WHtR3	3.3.3
Waist-to-height ratio 4	--	WHtR4	3.3.3
Whole and Regional Body Composition			
Whole body fat	kg	Whole BF	3.3.1
Whole percentage of body fat	%	Whole %BF	3.3.1
Whole fat free mass	kg	Whole FFM	3.3.1
Whole percentage of fat free mass	%	Whole %FFM	3.3.1
Trunk body fat	kg	Trunk BF	3.3.1
Trunk percentage of body fat	%	Trunk %BF	3.3.1
Trunk fat free mass	kg	Trunk FFM	3.3.1
Trunk percentage of fat free mass	%	Trunk %FFM	3.3.1
Abdominal body fat	kg	Abd BF	3.3.1
Abdominal percentage of body fat	%	ABD %BF	3.3.1
Central Abdominal body fat	kg	CAbd BF	3.3.1
Central Abdominal percentage of body fat	%	CAbd %BF	3.3.1

Descriptive statistics are presented as mean ± sd and range, for all analyzed variables. The Gaussian distribution of the data was assessed with the Shapiro-Wilk goodness-of-fit test. Paired-samples T test was used to compare the magnitude of the different WHtR mean values. Partial correlations, controlling for age and sex, and partial and part, also called semipartial (357), correlations, controlling for age and sex and BMI, were performed to assess the relations between dependent and independent variables. Only correlation coefficients equal or above 0.50 were considered to be significant at a significance level of 5% and a statistical power of 80% (357). Pairs of

correlation coefficients obtained between each WHtR with each dependent variable were compared, using Z statistic, to analyze if any WHtR was more closely associated with whole and central BF. Statistical calculations were performed using the IBM SPSS Statistics version 19 (SPSS, inc, Chicago, IL, USA), except for z statistic which was performed using Medcalc version 11.1.1.0 (MedCalc Software, Mariakerke, Belgium).

6.3. Results

Mean values for all studied variables are presented in table 6.2. From among the 28 studied NAFLD patients WHtR above the boundary value of 0.5 was present in nearly 100% of the sample, depending on the WCmp used. Results for WC measurements were considered to be different between all studied WCmp (WC4>WC2>WC3>WC1) and the magnitude of WHtR mean values were also different according to the WC used. Obesity was present in 9 subjects (3 were female), according to BMI classification, with no differences between sexes in mean BMI (p=0.075 on independent samples t test).

Table 6.3 shows the results for partial correlations controlled for age and sex, as well as partial and semipartial correlations, controlled for sex, age and BMI, between each WHtR and each whole or central studied BF depot. All but WHtR1 were correlated with all studied BF depots, showing coefficients of correlation magnitudes that were most often above 0.5, in partial correlations controlled only for age and sex. When BMI was added as a control variable in partial correlations, all WHtR were no longer correlated with whole BF and %BF, and WHtR4 was also not correlated with Trunk %BF. In semipartial correlations, controlled for age, sex and BMI, all WHtR were only correlated only with both absolute and relative values of abdominal (Abd) BF and central abdominal (CAbd) BF, except for WHtR4 which was not correlated with Abd %BF.

Table 6.2 – Descriptive data of the studied sample in study 3

	NAFLD Patients (n=28)			
Variables	**Mean ± sd ***		**Min. – Max.**	
Age, yr (median, yr)	49.5 ± 12.8	(49)	25 – 68	
Sex, n female (% female)	9	(32.1)		
Anthropometry				
Weight, kg (COV, %)	87.6 ± 12.7	(0.07)	66.2 – 115.8	
Height, cm (COV, %)	167.2 ± 9.2	(0.03)	149.5 – 183.7	
BMI, kg/m^2 (% obese)	29.1 ± 4.0	(32.1)	22.6 – 42.2	
WC 1, cm (COV, %)	100.7 ± 8.2#	(0.45)	86.0 – 119.8	
WC 2, cm (COV, %)	104.8 ± 10.6#	(0.49)	85.3 – 128.7	
WC 3, cm (COV, %)	103.7 ± 10.4#	(0.47)	85.7 – 129.3	
WC 4, cm (COV, %)	106.3 ± 11.7#	(0.73)	86.7 – 129.1	
WHtR 1 (≥0.5, %)	0.60 ± 0.07†	(96.4)	0.48 – 0.75	
WHtR 2 (≥0.5, %)	0.63 ± 0.08†	(100.0)	0.50 – 0.82	
WHtR 3 (≥0.5, %)	0.62 ± 0.08†	(96.4)	0.49 – 0.81	
WHtR 4 (≥0.5, %)	0.64 ± 0.09†	(100.0)	0.50 – 0.85	
Whole and Regional Body Composition				
Whole BF, kg (%)	27.2 ± 9.3	(31.31 ± 8.20)	13.7 – 51.2	(18.84 – 46.28)
Whole FFM, kg (%)	58.7 ± 9.1	(68.69 ± 8.20)	39.6 – 77.7	(53.72 – 81.16)
Trunk BF, kg (%)	15.2 ± 5.2	(33.15 ± 7.65)	7.4 – 25.0	(20.87 – 48.01)
Trunk FFM kg (%)	29.9 ± 3.9	(66.85 ± 7.65)	21.1 – 38.6	(51.99 –79.13)
Append BF, kg (%)	10.8 ± 4.8	(30.42 ± 10.39)	5.2 – 25.7	(13.63 – 50.40)
Append FFM, kg (%)	24.5 ± 5.1	(69.58 ± 10.39)	14.9 – 34.8	(49.60 – 86.37)
Abdominal BF, kg (%)	3.5 ± 1.2	(37.57 ± 6.59)	1.7 – 6.3	(26.09 – 49.40)
Central Abdominal BF, kg (%)	2.9 ± 0.8	(35.82 ± 5.70)	1.6 – 5.0	(24.28 – 44.64)

COV – coefficient of variation; BMI – body mass index; WC1 – Waist circumference as measured at minimal wait; WC2 - Waist circumference as measured just above iliac crest; WC3 - Waist circumference as measured at mid distance between lowest rib and iliac crest; WC4 - Waist circumference as measured at the umbilicus; WHtR1 – waist-to-height ratio using WC1; WHtR2 – waist-to-height ratio using WC2; WHtR3 – waist-to-height ratio using WC3; WHtR4 – waist-to-height ratio using WC4; BF – body fat; FFM – fat free mass; * Results are presented as mean ± standard deviation, unless otherwise noted; Min. – lowest observed value; Max. – highest observed value; BMI – body mass index; BF – body fat; FFM – fat free mass; # - different from all other WC mean values, $p < 0.05$ in paired samples t-test; † - different from all other WHtR mean values, $p < 0.05$ in paired samples t-test.

Table 6.3 – Partial and semipartial correlations between all studied waist-to-height ratios and body fat content variables.

Variables		Whole BF	Trunk BF	Abd BF	C Abd BF	Whole %BF	Trunk %BF	Abd %BF	C Abd %BF
WHtR 1	†	0.49	0.63*	0.81*	0.72*	0.51*	0.56*	0.65*	0.63*
	§	0.41	0.58*	0.80*	0.72*	0.45	0.51*	0.66*	0.63*
	¶	0.22	0.38*	0.70*	0.69*	0.22	0.32	0.54*	0.55*
WHtR 2	†	0.61*	0.73*	0.82*	0.74*	0.56*	0.59*	0.61*	0.61*
	‡	0.48	0.64*	0.84*	0.77*	0.46	0.52*	0.66*	0.63*
	#	0.26	0.43	0.74*	0.74*	0.23	0.32	0.54*	0.55*
WHtR 3	†	0.60*	0.72*	0.83*	0.74*	0.55*	0.59*	0.62*	0.61*
	‡	0.48	0.64*	0.84*	0.76*	0.46	0.52*	0.66*	0.62*
	#	0.25	0.42	0.74*	0.73*	0.22	0.32	0.54*	0.54*
WHtR 4	†	0.59*	0.68*	0.76*	0.68*	0.51	0.53*	0.56*	0.57*
	‡	0.44	0.58*	0.78*	0.71*	0.42	0.45	0.62*	0.60*
	#	0.23	0.38	0.68*	0.67*	0.20	0.27	0.49	0.50*

WHtR 1 – Waist-to-height ratio calculated using waist circumference measured at narrowest torso; WHtR 2 - Waist-to-height ratio calculated using waist circumference measured at iliac crest; WHtR 3 - Waist-to-height ratio calculated using waist circumference measured at midpoint between lowest rib and iliac crest; WHtR 4 - Waist-to-height ratio calculated using waist circumference measured at the umbilicus; BF – body fat; Trunk BF – Trunk body fat; Abd BF – Abdominal body fat; C Abd BF – Central abdominal body fat; † - partial correlations between studied WHtR and dependent variables, controlled for age and sex; ‡ - partial correlations between studied WHtR and dependent variables, controlled for age, sex and BMI; # - semipartial correlations between studied WHtR and dependent variables, adjusted for age, sex and BMI; * - significant for $p<0.05$ and $\beta=0.20$.

Table 6.4 shows the results for the comparison (p-values) between pairs of competing WHtR coefficients of correlation with each dependent variable. No differences were found between all compared coefficients of correlation.

6.4. Discussion

To our knowledge this is the first report to focus on the strength of correlation between WHtR and BF in NAFLD patients, and on the variation of such relation associated to different WCmp used to calculate WHtR. Mean WHtR was reasonably high and the prevalence of elevated WHtR, considering the 0.5 boundary value, was very high in the present sample. This was expected since it has been shown that NAFLD patients have higher values of WHtR (252). The magnitude of WHtR mean values were different according to the WC used in its calculation (WHtR4 > WHtR2 > WHtR3 > WHtR1) meaning they are not interchangeable. This may have large implications in clinical practice and data collection for longitudinal assessment of subjects (pre - post) as well as between groups comparisons

Table 6.4 – Z statistic P values for the comparison between the coefficients of correlation obtained in partial and semipartial correlation between the studied waist-to-height ratios and all dependent variables.

		WHtR 1		WHtR 2		WHtR 3		WHtR 4			
		p†	p‡	p†	p‡	p†	p‡	p†	p‡		
				0.975	0.991	0.989	0.997	0.892	0.929	Whole %BF	WHtR 1
				0.989	0.994	0.969	0.984	0.758	0.856	Trunk %BF	
				0.995	1.000	0.995	0.996	0.806	0.802	Abd %BF	
				0.981	0.988	0.991	0.992	0.861	0.841	C Abd %BF	
WHtR 2	Whole BF	0.731	0.872			0.986	0.994	0.868	0.920	Whole %BF	WHtR 2
	Trunk BF	0.717	0.856			0.981	0.991	0.747	0.850	Trunk %BF	
	Abd BF	0.663	0.799			0.990	0.996	0.801	0.802	Abd %BF	
	C Abd BF	0.705	0.740			0.972	0.980	0.843	0.830	C Abd %BF	
WHtR3	Whole BF	0.790	0.902	0.938	0.970			0.882	0.926	Whole %BF	WHtR 3
	Trunk BF	0.740	0.866	0.976	0.990			0.729	0.840	Trunk %BF	
	Abd BF	0.646	0.793	0.981	0.994			0.811	0.805	Abd %BF	
	C Abd BF	0.743	0.775	0.960	0.963			0.870	0.849	C Abd %BF	
WHtR4	Whole BF	0.880	0.964	0.847	0.907	0.908	0.937				
	Trunk BF	0.983	0.980	0.701	0.837	0.724	0.847				
	Abd BF	0.716	0.872	0.538	0.677	0.522	0.671				
	C Abd BF	0.948	0.900	0.658	0.647	0.695	0.680				

WHtR 1 – Waist-to-height ratio calculated using waist circumference measured at minimal waist; WHtR 2 - Waist-to-height ratio calculated using waist circumference measured at iliac crest; WHtR 3 - Waist-to-height ratio calculated using waist circumference measured at midpoint between lowest rib and iliac crest; WHtR 4 - Waist-to-height ratio calculated using waist circumference measured at the umbilicus; BF – body fat; Trunk BF – Trunk body fat; Abd BF – Abdominal body fat; C Abd BF – Central abdominal body fat; † - comparison between correlation coefficients obtained in partial correlations between different WHtR and all dependent variables, controlled for age, sex and BMI; ‡ - comparison between correlation coefficients obtained in semipartial correlations between different WHtR and all dependent variables, removing the effect of age, sex and BMI.

and, most important, subjects results confrontation with normative charts and cut-off or boundary values because dichotomous analysis may amplify even small absolute differences (365). Several previous studies also reported WC magnitudes (the changeable component of WHtR) to be influenced by WCmp (365-367). Still it have been advocated that current WC thresholds, generalized using WHO protocol (at the midpoint between lowest rib and iliac crest), could be applied to NIH measurements (at the superior border of the iliac crest) (23) because of small or absent differences found between measurements using these WCmp, particularly in men (365, 366). Even though we found differences between all studied WHtR mean values, both WHtR1 and WHtR3 only misclassified 1 subject (3.6%) at elevated risk as compared to WHtR2 and WHtR4, when a dichotomous approach was applied based on the boundary value of 0.5. Though this was not the aim of the present study, WHtR2 and WHtR4 may be considered preferable for diagnosing increased WHtR, considering they

were able to identify 100% of the studied NAFLD patients, which is a population with increased cardiovascular risk. This may suggest high sensitivity of WHtR for detecting patients with NAFLD though both WHtR' sensitivity and specificity for detecting NAFLD needs to be properly assessed and warrants further investigation.

In the present sample of NAFLD patients WHtR was highly associated with whole and central BF, controlled for age and sex. But when BMI was introduced as a control variable, WHtR was only correlated with central BF, meaning BMI overlaps the explanation of whole BF accounted by the variation of WHtR. When semipartial correlations were used, controlled for sex, age and BMI, WHtR were only correlated with absolute and relative values of the studied abdominal fat depots, meaning that control variables were explaining part of the variation of both trunk BF and trunk %BF that could not be explained by WHtR. Correlation coefficient magnitudes were particularly high for central BF depots, but WHtR4 correlations seem to be somewhat less consistent than those observed for the other WHtRs and therefore may be considered disadvantageous, as compared to the others. WHtR2 and WHtR3 seem slightly more consistently associated with NAFLD patients body composition (BC) than the other, but differences are minor. The association of WHtR with BC, particularly with central BF, has been reported in diverse groups (278, 279) but not until now in NAFLD patients. WHtR was also shown to predict higher cardiometabolic risk better than WC and BMI (129). The present study showed consistent coefficients of correlation of WHtR and central fat depots, even after controlling for age, sex and BMI, suggesting that WHtR explains the variation of abdominal fat far beyond BMI. Abdominal adipose tissue is known to be highly related to metabolic abnormalities and increased cardiometabolic risk, therefore it is reasonable to assume that WHtR may be highly related to the same metabolic abnormalities and increased cardiometabolic risk, in NAFLD, as already observed in other subpopulations, thought such hypothesis needs confirmation.

Comparisons between pairs of competing WHtR correlations results with each dependent variable showed that all studied WHtR are similarly associated with the analyzed BF depots, irrespectively of the WC used for its calculation. Previous studies have already found no differences in the association of WC alone, measured at different sites, with BF depots (366, 367). In a sound review, by Ross and colleagues (23), it was concluded that the use of different WCmp did not changed the well-established relationships between WC and morbidity of cardiovascular disease and diabetes and with cardiovascular and all-cause mortality. Since WHtR have proven highly sensitive in the prediction of cardiovascular risk (129), the absence of WCmp influence in risk prediction should be confirmed in future studies, using WC measured at different sites, to calculate WHtR.

The present study confirms the strong association between WHtR and BF, specially central BF, even after controlling for age, sex and BMI, in NAFLD patients. No change in the strength of relation between WHtR and both whole and central BF depots was found in the present study, regardless of the WCmp used, and a preferable WCmp to calculate WHtR could not be clearly established. The results of the present study may endorse an interchangeable use of different WCmp to identify subjects above boundary value and therefore at higher risk. Additional research is needed to confirm the influence of different WCmp on the variation of WHtR associations with cardiometabolic risk factors, in NAFLD patients.

Chapter 7 – Study 4:

"Does Waist Circumference Measurement Protocol Used Influences the Relation Between Waist-to-hip Ratio and Body Fat Content and Distribution in Non-Alcoholic Fatty Liver Disease Patients?"[4]

[4] Pimenta, N, Santa-Clara, H, Cortez-Pinto, H, Silva-Nunes, J, Sardinha, L (2013) Clinical Nutrition (Submitted).

Abstract

Background: Body Fat (BF), particularly central fat accumulation, is related to Non-alcoholic Fatty Liver Disease (NAFLD), and is related also to increased cardiovascular morbidity and mortality, which is also increased in NAFLD patients. Waist-to-hip ratio (WHR) has been suggested to be a clinical marker of BF distribution capable of diagnosing adverse and pathogenic BF distribution profiles. No single waist circumference measurement protocol (WCmp) is consensually accepted nor its effects on WHR correlation have been assessed.

Purpose: The aim of this study was to analyze whether the most used WCmp affect the strength of association between WHR and BF content and BF distribution, in NAFLD patients.

Methods: Whole and regional BF content were assessed with Dual Energy X-ray Absorptiometry (DXA) in 28 NAFLD patients (19 males, 51 ± 13 yrs, and 9 females, 47 ± 13 yrs) , who were diagnosed by liver biopsy or ultrasound, after exclusion of other potential causes of liver disease. Ratios between DXA assessed BF depots were used to assess BF distribution. Anthropometric evaluation included the measurement of WC using four different WCmp (WC1: minimal waist; WC2: iliac crest; WC3: mid-distance between iliac crest and lowest rib; WC4: at the umbilicus) and WHR was calculated using each of the WC measurements (WHR1, WHR2, WHR3 and WHR4, respectively).

Results: No correlations were found with whole BF, trunk BF and appendicular BF. Only WHR2, WHR3 and WHR4 were correlated with Abdominal BF (r=0.59; r=0.59; r=0.58; respectively), Central Abdominal BF (r=0.61; r=0.60; r=0.58; respectively), trunk-to-appendicular BF (r=0.56; r=0.56; r=0.51; respectively) and Abdominal BF-to-whole BF ratio (r=0.51; r=0.53; r=0.54; r=0.53; respectively), controlled for age, sex and body mass index.

Conclusions: The present study confirms the strong relation of WHR and a central distribution of BF, regardless of WCmp used, in NAFLD patients. WHR1 seemed, at least, less consistent as a central BF surrogate. WHR2 and WHR3 may used interchangeably for the diagnosis of high WHR and, may be considered preferable for use in clinical practice, for the calculation of NAFLD patients WHR.

Keywords: Body Fat; Hepatic Steatosis; Body Composition; Anthropometry, body fat distribution.

7.1. Introduction

Body composition (BC) has an important role in the etiology of non-alcoholic fatty liver disease (NAFLD) (9). Both central body fat (BF) and insulin resistance have been found to increase the risk of NAFLD (363), yet whole and particularly central BF may further increase the risk for NAFLD by its strong association with insulin resistance (5, 183), as well as with other disturbances (60-65) Accordingly waist-to-hip ratio (WHR) was shown to be closely related to the occurrence of NAFLD (252). WHR is assumed to reflect the distribution of fat throughout the body and a high WHR should represent a preferential abdominal or central accumulation of BF (128) and it has generally proven to be so (91, 138, 139) yet conflicting results have also been found (263). WHR was initially suggested to be calculated using measurements of waist circumference (WC) at the minimal waist (261) but WC measured at the mid distance between lowest rib and iliac crest has been the most used protocol (179). The considerable variation in methodology and results found in the literature may be limiting a wider usage of this body index. The importance of using one consensual standardized measurement protocol to calculate WHR was recognized (264, 265). Yet no single consensual measurement protocol in consensually accepted and a wide diversity of methods for the measurement and calculus of WHR can be found in the literature (179).

To our knowledge it is unknown if the use of different commonly used WC, with different measuring sites, affect the relation between WHR and both whole and central BF content and BF distribution. Therefore the aim of the present study was to analyze which of the most used WC measurement protocol (WCmp) is better to calculate WHR for use in clinical practice with NAFLD patients as a surrogate of whole and central BF content and BF distribution.

7.2. Methods

In the present study we analyzed two groups of variables (Table 7.1): whole and regional BF content, as assessed by DXA (as described in subsections 3.3.1); BF distribution assessed using ratios between different DXA assessed BF depots (as described in subsections 3.3.2) and WHR as calculated using four different WC measured according to different WCmp for each patient (as detailed in subsection 3.3.3), in the studied sample of 28 NAFLD patients (described in section 3.1).

Table 7.1 – List of Studied variables in study 4

Variable	Unit of measurement	Abbreviations	Described in subsection
Age	Years	--	3.1
Sex	--	--	3.1
Anthropometry			
Weight	kg	--	3.3.3
Height	cm	--	3.3.3
Body mass index	kg/m^2	BMI	3.3.3
Waist Circumference 1	cm	WC1	3.3.3
Waist Circumference 2	cm	WC2	3.3.3
Waist Circumference 3	cm	WC3	3.3.3
Waist Circumference 4	cm	WC4	3.3.3
Waist-to-hip ratio 1	--	WHR1	3.3.3
Waist-to-hip ratio 2	--	WHR2	3.3.3
Waist-to-hip ratio 3	--	WHR3	3.3.3
Waist-to-hip ratio 4	--	WHR4	3.3.3
Whole and Regional Body Composition			
Whole body fat	kg	Whole BF	3.3.1
Whole percentage of body fat	%	Whole %BF	3.3.1
Trunk body fat	kg	Trunk BF	3.3.1
Trunk percentage of fat mass	%	Trunk %BF	3.3.1
Appendicular body fat	kg	Append BF	3.3.1
Appendicular percentage of fat mass	%	Append %BF	3.3.1
Abdominal body fat	kg	Abd BF	3.3.1
Abdominal percentage of body fat	%	ABD %BF	3.3.1
Central Abdominal body fat	kg	CAbd BF	3.3.1
Central Abdominal percentage of body fat	%	CAbd %BF	3.3.1
Body fat distribution variables			
Trunk BF-to-Appendicular BF	--	--	3.3.2
Abdominal BF-to-whole BF	--	--	3.3.2
Abdominal BF-to-trunk BF	--	--	3.3.2

Descriptive statistics are presented as mean ± sd and range for all analyzed variables. The Gaussian distribution of the data was assessed with the Shapiro-Wilk goodness-of-fit test. Paired-samples T test was used to compare the magnitude of the different WHR mean values. Partial and part, also called semipartial (357), correlations, controlling for age and sex and body mass index (BMI), were performed to assess the relations between dependent and independent variables. Only correlation coefficients equal or above 0.50 were considered to be significant at a significance level of 5% and a statistical power of 80% (357). Pairs of correlation coefficients obtained between each WHR with each dependent variable were compared, using Z statistic, to analyze if any WHR was

more closely associated with whole and central BF. Statistical calculations were performed using the IBM SPSS Statistics version 19 (SPSS, inc, Chicago, IL, USA), except for z statistic which was performed using Medcalc version 11.1.1.0 (MedCalc Software, Mariakerke, Belgium).

7.3. Results

Mean values for all studied variables are presented in table 7.2. High WHR was present in about 70 to over 80%, according to the WHR used, yet WHR2 and WHR3 diagnosed exactly the same prevalence of high WHR in the studied sample. Results for WHR were considered to be different when using different WC (WHR4>WHR2>WHR3>WHR1).

The results for partial and semipartial correlations, controlled for sex and age (table 7.3), showed all WHR to be preferentially correlated with central BF. Partial correlations, controlled for age and sex, show WHR1 to only correlated only with abdominal BF-to-whole BF ratio. All other studied WHR correlate also with abdominal (Abd) BF, central abdominal (CAbd) BF and trunk BF-to-appendicular BF ratio. However, the only significant coefficients of correlation found in semipartial correlations, also controlled only for sex and age, were found between WHR1, WHR2 and WHR3 and both Abd BF and CAbd BF.

The results for partial and semipartial correlations, controlled for sex, age and BMI (table 7.4), showed that only WHR1, WHR2 and WHR3 were correlated with any of the studied BF depots or BF distribution ratios. WHR 2,WHR 3 and WHR4 were all correlated with Abd BF, CAbd BF, trunk BF-to-appendicular BF ratio and abdominal BF-to-whole BF ratio, in partial correlations controlled for age, sex and BMI. In semipartial correlations, controlled also for age, sex and BMI, WHR1, WHR2 and WHR3 were only found to be associated with Abd BF and CAbd BF.

Coefficients of correlation tended to increase when BMI was added as control variable, even in semipartial correlations, but this increase was not significant ($p>0.05$ in all coefficient of correlation comparisons by Z statistics, using Medcalc version 11.1.1.0 [MedCalc Software, Mariakerke, Belgium], data not shown). All coefficients of correlation remained relatively stable either when using partial as in semipartial correlations. Also no differences were found in the comparison between any pair of coefficients of correlation obtained between competing WHR with each dependent variable (listed in table 7.3 and 7.4), using Z statistics ($p>0.05$ in all comparisons, data not shown).

Table 7.2 – Descriptive data of the studied sample in study 4.

	NAFLD Patients (n=28)			
Variables	**Mean ± sd ***		**Min. – Max.**	
Age, yr (median, yr)	49.5 ± 12.8	(49)	25 – 68	
Sex, n female (% female)	9	(32.1)		
Anthropometry				
Weight, kg (COV, %)	87.6 ± 12.7	(0.07)	66.2 – 115.8	
Height, cm (COV, %)	167.2 ± 9.2	(0.03)	149.5 – 183.7	
BMI, kg/m^2 (% obese)	29.1 ± 4.0	(32.1)	22.6 – 42.2	
WC 1, cm (COV, %)	100.7 ± 8.2#	(0.45)	86.0 – 119.8	
WC 2, cm (COV, %)	104.8 ± 10.6#	(0.49)	85.3 – 128.7	
WC 3, cm (COV, %)	103.7 ± 10.4#	(0.47)	85.7 – 129.3	
WC 4, cm (COV, %)	106.3 ± 11.7#	(0.73)	86.7 – 129.1	
Hip-C, cm (COV, %)	107.6 ± 12.0	(0.38)	92.3 – 138.3	
WHR 1 (high WHR, %)	0.94 ± 0.07†	(69.2)	0.75 – 1.03	
WHR 2 (high WHR, %)	0.98 ± 0.07†	(76.9)	0.85 – 1.11	
WHR 3 (high WHR, %)	0.97 ± 0.07†	(76.9)	0.82 – 1.09	
WHR 4 (high WHR, %)	0.99 ± 0.06†	(84.6)	0.88 – 1.10	
Whole and Regional Body Fat				
Whole BF, kg (%)	27.2 ± 9.3	(31.31 ± 8.20)	13.7 – 51.2	(18.84 – 46.28)
Trunk BF, kg (%)	15.2 ± 5.2	(33.15 ± 7.65)	7.4 – 25.0	(20.87 – 48.01)
Append BF, kg (%)	10.8 ± 4.8	(30.42 ± 10.39)	5.2 – 25.7	(13.63 – 50.40)
Abdominal BF, kg (%)	3.5 ± 1.2	(37.57 ± 6.59)	1.7 – 6.3	(26.09 – 49.40)
Central Abdominal BF, kg (%)	2.9 ± 0.8	(35.82 ± 5.70)	1.6 – 5.0	(24.28 – 44.64)
Body Fat distribution, ratios				
Trunk BF/ Append BF ratio	1.477 ± 0.371		0.958 – 2.547	
Abdominal BF / BF ratio	0.130 ± 0.025		0.045 – 0.185	
Abdominal BF / Trunk BF ratio	1.231 ± 0.039		0.095 – 0.299	

COV – coefficient of variation; BMI – body mass index; WC1 – minimal waist circumference measured at the narrowest part of the torso; WC2 – waist circumference measured just above the iliac crest; WC3 – waist circumference measured at the mid-distance between lowest rib and iliac crest; WC4 - Waist circumference at the umbilicus; WHR 1 – waist-to-hip ratio using WC1; WHR 2 – waist-to-hip ratio using WC2; WHR 3 – waist-to-hip ratio using WC3; WHR 4 – waist-to-hip ratio using WC4; BF – body fat; FFM – fat free mass; * Results are presented as mean ± standard deviation, unless otherwise noted; Min. – lowest observed value; Max. – highest observed value; BMI – body mass index; BF – body fat; FFM – fat free mass; # - different from all other WC mean values, $p < 0.05$ in paired samples t-test; † - different from all other WHR mean values, $p < 0.05$ in paired samples t-test.

Table 7.3 – Partial and semipartial correlations between all studied waist-to-hip ratios and body composition, controlled for age and sex.

Variables	WHR1		WHR2		WHR3		WHR4	
	†	§	†	§	†	§	†	§
Whole BF	- 0.17	- 0.12	0.13	0.09	0.09	0.06	0.10	0.07
Whole %BF	- 0.02	- 0.02	0.15	0.08	0.13	0.07	0.12	0.06
Trunk BF	0.05	0.04	0.35	0.27	0.31	0.25	0.31	0.25
Trunk %BF	0.12	0.08	0.29	0.19	0.29	0.19	0.24	0.16
Append BF	- 0.42	- 0.28	- 0.16	- 0.11	- 0.21	- 0.13	- 0.17	- 0.11
Append %BF	- 0.18	- 0.08	- 0.06	- 0.03	- 0.08	- 0.04	- 0.05	- 0.02
Abd BF	0.37	0.33	0.57*	0.51*	0.56*	0.50*	0.57*	0.51*
Abd %BF	0.34	0.28	0.44	0.36	0.44	0.36	0.42	0.35
CAbd BF	0.40	0.39	0.60*	0.58*	0.58*	0.56*	0.57*	0.56*
CAbd %BF	0.25	0.22	0.40	0.32	0.36	0.31	0.34	0.30
Trunk BF/ Append BF	0.45	0.37	0.56*	0.46	0.56*	0.46	0.51*	0.42
Abdominal BF/ whole BF	0.52*	0.47	0.51*	0.46	0.54*	0.49	0.52*	0.47
Abdominal BF/ Trunk BF	0.38	0.37	0.30	0.29	0.32	0.32	0.33	0.33

WHR 1 – Waist-to-Hip ratio calculated using waist circumference measured at narrowest torso; WHR 2 - Waist-to-Hip ratio calculated using waist circumference measured at iliac crest; WHR 3 - Waist-to-Hip ratio calculated using waist circumference measured at midpoint between lowest rib and iliac crest; WHR 4 - Waist-to-Hip ratio calculated using waist circumference measured at the umbilicus; BF – body fat; Trunk BF – Trunk body fat; Abd BF – Abdominal body fat; CAbd BF – Central abdominal body fat; † - partial correlations between studied WHR and dependent variables, controlled for age and sex; § - semipartial correlations between studied WHR and dependent variables, controlled for age and sex ; * - significant for $p<0.05$ and $\beta=0.20$.

Table 7.4 – Partial and semipartial correlations between all studied waist-to-hip ratios and body composition, controlled for age, sex and BMI.

Variables	WHR1		WHR2		WHR3		WHR4	
	†	§	†	§	†	§	†	§
Whole BF	0.06	0.03	0.19	0.13	0.18	0.10	0.15	0.08
Whole %BF	0.13	0.06	0.17	0.08	0.18	0.09	0.13	0.06
Trunk BF	0.29	0.19	0.43	0.28	0.43*	0.28	0.38	0.25
Trunk %BF	0.27	0.17	0.33	0.20	0.34	0.21	0.26	0.16
Append BF	- 0.29	- 0.14	- 0.19	- 0.09	- 0.20	- 0.10	- 0.22	- 0.11
Append %BF	- 0.08	- 0.03	- 0.06	- 0.02	- 0.05	- 0.02	- 0.05	- 0.02
Abd BF	0.47	0.41	0.59**	0.52**	0.59**	0.52**	0.58**	0.51**
Abd %BF	0.39	0.32	0.45	0.37	0.44	0.37	0.42	0.35*
CAbd BF	0.48	0.46	0.61**	0.58**	0.60**	0.58**	0.58**	0.56**
CAbd %BF	0.31	0.27	0.37	0.32	0.37	0.32	0.35	0.30
Trunk BF/ Append BF	0.45	0.38	0.56**	0.46	0.56**	0.46	0.51*	0.42
Abdominal BF/ whole BF	0.48	0.43	0.510*	0.46	0.53**	0.48	0.53*	0.47
Abdominal BF/ Trunk BF	0.32	0.30	0.300	0.28	0.32	0.30	0.34	0.32

WHR 1 – Waist-to-Hip ratio calculated using waist circumference measured at narrowest torso; WHR 2 - Waist-to-Hip ratio calculated using waist circumference measured at iliac crest; WHR 3 - Waist-to-Hip ratio calculated using waist circumference measured at midpoint between lowest rib and iliac crest; WHR 4 - Waist-to-Hip ratio calculated using waist circumference measured at the umbilicus; BF – body fat; Trunk BF – Trunk body fat; Append – Appendicular; Abd BF – Abdominal body fat; C Abd BF – Central abdominal body fat; † - partial correlations between studied WHR and dependent variables, controlled for age, sex and BMI; § - semipartial correlations between studied WHR and dependent variables, controlled for age, sex and BMI; * - significant for $p<0.05$ and $\beta=0.20$.

7.4. Discussion

Mean WHR was somewhat high and the prevalence of elevated WHR, was also rather high in the present sample. This was expected as NAFLD patients have shown to have higher values of WHR (252). The differences observed in the magnitude of WHR mean values, calculated using different WC, suggest they are not interchangeable, which may have important implications in clinical practice and data collecting, advising a careful and consistent choice of the WCmp used for the calculation of WHR. But the literature is neither consistent nor consensual regarding which WCmp should be used for the calculation of WHR. Several previous studies have reported WC magnitudes to be influenced by WCmp (365, 366). Still it has been suggested that WC measured at the midpoint between lowest

rib and iliac crest or at the superior border of the iliac crest could be used interchangeably for the diagnosis of obesity (23). Even though the present study shows differences between all WHR, both WHR2 and WHR3 diagnosed exactly the same prevalence of high WHR, using recommended cut-off values (261), suggesting they could be used interchangeably, in the present sample, for the diagnosis of high WHR.

The results concerning the correlations with whole and regional BF content suggests that WHR1 (calculated using WC measured at minimal waist) cannot be accepted as a proper surrogate. Albeit WHR was initially suggested to be calculated using WC measured at minimal waist (261), this was the least useful protocol in the present study. WHR2, WHR3 and WHR4 had a similar performance in the relation with the studied BF content variables. These WHRs were found to be particularly related with central BF, in the studied NAFLD patients. Though not consensually, the association between WHR and central BF was previously reported (91, 139) but not until now in NAFLD patients. Adding BMI as a control variable had little or no effect on the coefficients of correlation of the three most useful WHR with both Abd BF and CAbd BF, either in partial as in semipartial correlations, meaning BMI does not relate to dependent variables beyond that already accounted by the other control variables (sex and age). In fact BMI has been suggested to be more related to overall BF (122, 123), particularly with whole and subcutaneous adipose tissue (124), and does not take into account regional BF deposition (140) which has been identified as an important correlate leading to CVD (13) and possibly to NAFLD (5, 16). Therefore, when assessing obesity, BMI may be deceptive (17). The present results underlies the assumption that WHR, particularly WHR1, WHR2 or WHR3, may be of utmost importance in the clinical assessment of BC of NAFLD patients. When semipartial, instead of partial, correlations were used, the coefficients of correlation between WHR2, WHR3 and WHR4 and both Abd BF and CAbd BF were fairly unchanged, meaning that three mentioned WHRs were strongly associated with the studied abdominal fat depots, regardless of age, sex and BMI. Considering that the studied abdominal BF depots have been shown to be closely related to visceral adipose tissue (79, 80) which, in turn, was found to be a risk factor for the presence of NASH (46), it is reasonable to suggest that WHR assessment may be fundamental in the clinical assessment of NAFLD patients. WHR, using WC measured at mid-distance, was shown to be positively correlated with abdominal adipose tissue compartments, as assessed by magnetic resonance imaging (MRI) in free-living men (139). Coefficients of correlation over 0.70 were also found between WHR, using WC measured at the umbilicus, and both visceral and subcutaneous adipose tissue, as assessed by MRI at the level of the fourth lumbar vertebra (L4) (91). Even though

these results are in accordance to the findings of the present study, there are considerable differences in the methodology, including considerable sample differences, therefore extended interpretations are restrained. Thus opposing results were also found both in men and women when correlating WHR, using WC measured at the umbilicus, with DXA assessed BF depots similar to the present study, including central BF ($r<0.28$) and peripheral BF ($r<0.16$) (263). But the mentioned contradictions may rely on significant sample differences, as compared to the present study. The mentioned study included only subjects in the mid-thirties with completely different health status (263). Bottom line, WHR1, WHR2 and WHR3 seem useful in the assessment of central BF accumulation, which is more strongly related with adverse outcomes than whole BF, and therefore considered to be useful alternatives in the assessment of BC of NAFLD patients, in clinical settings.

When focusing on BF distribution, all WHR were positively correlated with BF distribution variables, as observed by the significant coefficients of correlation of all studied WHR with Abdominal BF-to-whole BF ratio, but only WHR2, WHR3 and WHR4 were correlated also with trunk BF-to-appendicular BF ratio, in partial correlations controlled for age and sex. As mentioned, BMI has been suggested to be more related to overall subcutaneous adipose tissue (124) and provides only a crude measurement of total adiposity (126, 130), therefore, it was not thoroughly surprising to find that adding BMI as controlling variable had a rather small impact on the studied coefficients of correlation, even though WHR1 was no longer related with any BF distribution variable, confirming the limited usefulness of the WHR calculated using minimal waist. Fully opposite results were found in other study, reporting high coefficients of correlation between WHR using minimal waist, and both DXA derived trunk-to-leg BF ratio ($r=0.62$) and waist-to-hip BF ratio ($r=0.78$) (138). The mentioned study was conducted on a sample of healthy white women which is profusely different form the sample analyzed in the present study. The results from the present study are in agreement with previous findings showing WHR, calculated using WC measured at the umbilicus, not correlated to trunk BF-to-appendicular BF ratio (263). Yet this was also conducted in a sample of adolescent girls. In semipartial correlations no significant associations were found between any studied WHR and any BF distribution variable. This underlies the importance of age and sex, more than BMI, in the variation of BF distribution, and raises questions about the usefulness of WHR as a BF distribution surrogate in NAFLD patients.

The present study confirms the strong association between WHR and central BF, even after controlling for age, sex and BMI, in NAFLD patients. However WHR using WC measured at minimal

waist does not seem useful as a BF content and/or distribution surrogate in NAFLD. In NAFLD patients WHR seems a good surrogate of only central BF and not of BF distribution, as has been advocated. The results of the present study may endorse an interchangeable use of WHR2 and WHR3 to identify subjects with high WHR and therefore at higher risk. Based on present results both WHR2 and WHR3 seem preferable to use in NAFLD patients. Additional research is needed to confirm the influence of different WCmp in the relation of WHR with other NAFLD and cardiometabolic risk factors.

Chapter 7 – Study 4: "Does Waist Circumference Measurement Protocol Influences the Relation Between Waist-to-Hip Ratio and Body Fat Content and Distribution in Non-Alcoholic Fatty Liver Disease Patients?"

Chapter 8 – Study 5:

"Are Body Indexes and Circumferences Useful Surrogates of Body Fat Content and Distribution in Non-Alcoholic Fatty Liver Disease Patients?"[5]

[5] Pimenta, N, Santa-Clara, H, Cortez-Pinto, H, Silva-Nunes, J, Sardinha, L (2013) Clinical Nutrition (submitted).

Abstract

Background: Body composition (BC), particularly central body fat (BF), is a major concern in Non-alcoholic Fatty Liver Disease (NAFLD). Body mass index (BMI) limitations are well known and several other body indexes are gaining importance.

Purpose: The aim of the present investigation was twofold: to analyze how body circumferences and indexes perform as surrogate of whole and regional BF content and BF distribution in NAFLD patients, and to find if any specific body index and/or circumference perform better than the commonly used BMI as surrogate of BC in NAFLD patients.

Methods: Absolut and relative whole BF, trunk BF, appendicular BF, abdominal (Abd) BF (measured between the upper edge of L2 and the lower edge of L4) and central abdominal (CAbd) BF (measured as abdominal BF but limited to the lateral sides of rib cage) were assessed with Dual Energy X-ray Densitometry (DXA) in 28 NAFLD patients (19 males, 51 + 13 yrs, and 9 females, 47 + 13 yrs), who were diagnosed through liver biopsy or ultrasound, after exclusion of other potential causes of liver disease. Body circumferences included waist (WC), hip (Hip-C), arm (Arm-C), thigh (Thigh-C) and calf (Calf-C) measurements, and indexes included the calculation of BMI, body adiposity index (BAI = [Hip-C / height^1.5]-18), Waist-to-height ratio (WHtR = WC / height) and waist-to-hip ratio (WHR=WC / Hip-C).

Results: Partial correlations showed that, whole BF was significantly associated with nearly all independent variables, including Arm-C (r=0.76), WC (0.79), Hip-C (r=0.88), Thigh-C (r=0.74), Calf-C (r=0.79), BMI (r=0.65), BAI (r=0.58) and WHtR (r=0.63), controlled for age and sex. However, when controlled also for BMI only WC was associated with Trunk BF (r=0.87); WC, WHtR and WHR were associated with Abd BF (r=0.78; r=0.84 and r=0.59, respectively) and CAdb BF (r=0.77; r=0.76 and r=0.61, respectively); WHtR was related with Abdominal BF-to-whole BF ratio and arm circumference was inversely related with abdominal BF-to-trunk BF ratio (r=-0.53), consistently. Only WC, WHtR and WHR were found better surrogates than BMI for some of the mentioned dependent variables.

Conclusions: Most body circumferences and both BMI and BAI were found limited in the assessment of both central BF accumulation and BF distribution. BMI is neither superior to other tested BF surrogates, nor independent of WC in the assessment of BF in NAFLD patients. The present study supports the inclusion WC and/or WHtR, as good alternative methods to BMI, to assess BC of NAFLD patients in clinical settings.

Keywords: Hepatic Steatosis; DXA, Body Composition; Anthropometry, Body Mass Index.

8.1. Introduction

Body composition (BC) has been suggested to have an important role in the etiology of non-alcoholic fatty liver disease (NAFLD) (9). Central body fat (BF) accumulation seems even more related to NAFLD and its related features than whole BF (5, 16, 46). Sound evidence has been showing the importance of centrally accumulated BF for the development of NAFLD (46, 60, 61).

NAFLD is a common cause of liver disease encompassing two basic histological lesions: (I) hepatic steatosis (fat accumulation in hepatocytes), and (II) non-alcoholic steatohepatitis (characterized by hepatic steatosis and inflammation along with a constellation of other disturbances) (30-32). Ultimately NAFLD can lead to advanced fibrosis, cirrhosis, liver failure and death (16, 30). NAFLD patients often present high levels of BF accumulation and obesity, as compared to the general population (44). BC has been suggested to have an important role in the etiology of NAFLD (9). Central BF accumulation seems even more related to NAFLD, and its related features, than whole BF (5, 16, 46). Sound evidence has been showing the importance of centrally accumulated fat for the development of NAFLD (46, 60, 61). BF, besides being an important independent risk factor for NAFLD, is also strongly associated with other risk factors for NAFLD, such as insulin resistance (5). Additionally, excess both whole and central BF accumulation, or central BF distribution, are also known cardiovascular risk factors.

Body mass index (BMI) is recommended to assess obesity status is general population (100) as well as in specific higher risk sub-populations (372) and has been widely used in NAFLD patients, along with other anthropometric measures (137, 175, 183, 252, 363). However BMI limitations have long been identified and are well known (21, 22) and other body indexes, such as waist-to-height ratio (WHtR) and waist-to-hip ratio (WHR) are gaining importance (24, 129, 252). Waist circumference (WC) has been suggested as a preferable method to determine obesity in coronary artery disease patients (373), however waist-to-height ratio has been recently shown to more strongly identify coronary risk in middle aged men (247). WHtR has also been suggested as a preferable central obesity marker in the general population, along with an appealing public health message: "keep your waist circumference to less than half your height" (129). A novel body index named Body Adiposity Index (BAI) was argued as a better index of adiposity (24). To our knowledge the relation of BMI and other clinical BC surrogates with whole and regional BC have not been tested in NAFLD patients. Recently WHR ratio was considered to be the most important tested BC marker

for the prediction NAFLD, as compared to BMI or waist-to height-ratio (WHtR), however the prediction of either whole or regional BC, as well as BF distribution, was not tested (252).

To our knowledge the usefulness of some body circumferences and indexes as surrogates of BF content and distribution in NAFLD patients, is unknown. BMI limitations are well known, yet no alternative have been largely accepted and BMI is still the reference method for the diagnosis of obesity and obesity related risks and is the most used BC marker in clinical practice. There are strong arguments supporting the routine use of BMI, however the BMI arguments may be common to several alternatives. Therefore the aim of the present investigation was twofold: (I) to analyze how body circumferences and indexes perform as surrogate of whole and regional BF content and BF distribution in NAFLD patients, and (II) to find if any specific body index and/or circumference perform better than the commonly used BMI as surrogate of BC in NAFLD patients.

8.2. Methods

In the present study we analyzed two groups of variables (Table 8.1): whole and regional BF content, as assessed by DXA (as described in subsections 3.3.1); BF distribution assessed using ratios between different DXA assessed BF depots (as described in subsections 3.3.2) and body indexes and circumferences (as detailed in subsection 3.3.3), in the studied sample of 28 NAFLD patients (described in section 3.1). In the present study, based on the results from the previous studies in the present thesis, we used WC assessed at the level of the iliac crest (102, 171, 189, 190). The mentioned was the WC used to calculate both WHtR and WHR.

Descriptive statistics are presented as mean ± sd and range for all analyzed variables. The Gaussian distribution of the data was assessed with the Shapiro-Wilk goodness-of-fit test. Partial and part, also called semipartial (357), correlations were performed to assess the relations between dependent and independent variables controlling for age, sex and BMI. When BMI was an independent variable the correlation was controlled for age, sex and WC. Considering the size of the studied sample, to attain a statistical power of 80% ($\beta = 0.20$) at a statistical significance level of 5% ($\alpha=0.05$), only coefficients of correlation equal or superior to 0.50, corresponding to a large effect size, were considered significant (357). To observe if any of the studied body circumferences or indexes performed better than the commonly used BMI as surrogate of whole and central BF content and BF distribution, in NAFLD patients, the correlation coefficients obtained between BMI and each

Table 8.1 – List of Studied variables in study 5

Variable	Unit of measurement	Abbreviations	Described in subsection
Age	Years	--	3.1
Sex	--	--	3.1
Anthropometry			
Weight	Kg	--	3.3.3
Height	Cm	--	3.3.3
Arm Circumference	Cm	Arm-C	3.3.3
Waist Circumference	Cm	WC	3.3.3
Hip Circumference	Cm	Hip-C	3.3.3
Thigh Circumference	Cm	Thigh-C	3.3.3
Calf Circumference	Cm	Calf-C	3.3.3
Body Indexes			
Body mass index	kg/m^2	BMI	3.3.3
Body Adiposity index	--	BAI	3.3.3
Waist-to-height ratio	--	WHtR	3.3.3
Waist-to-hip ratio	--	WHR	3.3.3
Whole and Regional Body Composition			
Whole body fat	Kg	Whole BF	3.3.1
Whole percentage of body fat	%	Whole %BF	3.3.1
Whole fat free mass	Kg	Whole FFM	3.3.1
Whole percentage fat free mass	%	Whole %FFM	3.3.1
Trunk body fat	Kg	Trunk BF	3.3.1
Trunk percentage of body fat	%	Trunk %BF	3.3.1
Trunk fat free mass	Kg	Trunk FFM	3.3.1
Trunk percentage fat free mass	%	Trunk %FFM	3.3.1
Appendicular body fat	Kg	Append BF	3.3.1
Appendicular percentage of body fat	%	Append %BF	3.3.1
Appendicular fat free mass	Kg	Append FFM	3.3.1
Appendicular percentage of fat free mass	%	Append %FFM	3.3.1
Abdominal body fat	Kg	Abd BF	3.3.1
Abdominal percentage of body fat	%	ABD %BF	3.3.1
Central Abdominal body fat	Kg	CAbd BF	3.3.1
Central Abdominal percentage of body fat	%	CAbd %BF	3.3.1
Body fat distribution variables			
Trunk BF-to-Append BF ratio	--	--	3.3.2
Abdominal BF-to-whole BF ratio	--	--	3.3.2
Abdominal BF-to-trunk BF ratio	--	--	3.3.2

dependent variable were compared with the coefficients of correlation obtained by all other Independent variable for the same dependent variable using Z statistic. The level of significance was set at $P<0.05$ (two-tailed). Statistical calculations were performed using the IBM SPSS Statistics

version 19 (SPSS, inc, Chicago, IL, USA), except for z statistic which was performed using Medcalc version 11.1.1.0 (MedCalc Software, Mariakerke, Belgium).

8.3. Results

Mean values for all studied variables are presented in Table 8.2. From among the 28 studied NAFLD patients, 78.6% had excessive weight, including 32.1 % who were obese, and high levels of WC, WHtR and WHR were present in 57.1 %, 100% and 67.8 % of the patients, respectively. Mean BMI of the studied sample was in the overweight category. BMI and WHR were not different between both sexes (p=0.067 and p=0.058 obtained in an independent samples t test comparison, respectively), but female patients had higher WC (112.1 cm *vs* 102.1 cm, p=0.030), Hip-C (120.7 cm *vs* 102.9 cm, p=0.014) and Tight (56.6 cm *vs* 51.5 cm, p=0.038) as well as BAI (43.2 *vs* 21.2, p<0.001) and WHtR (0.71 *vs* 0.60, p<0.001). In Pearson correlation WHR (r=0.543, p=0.004), arm (Arm-C) (r=-0.476, p=0.014), thigh (Thigh-C) (r=0.713, p<0.001) and calf (Calf-C) (r=-0.540, p=0.004) circumferences were associated with patients' age.

Table 8.3 shows the results for partial and semipartial correlations between each independent variable (clinical markers of BF content and distribution) and each dependent variable (DXA assessed BF content and distribution), controlled for sex and age. All but WHR are associated with whole BF and trunk BF in partial correlations controlled for age and sex. Appendicular BF was only consistently predicted by Hip-C, Thigh-C and Arm-C. Other markers were also associated with appendicular BF but these were critically affected by age and sex, as observed in semipartial correlation results. BF in the abdomen seems to be particularly associated to WC, WHtR and WHR, but only WHtR showed a strong association with all absolute and relative variables from both abdominal (Abd) BF and central abdominal (CAbd) BF, unaffected by age and sex. Both BAI was also somewhat correlated with Abd BF, controlled for age and sex.

A consistent inverse relation between arm-C and Abd BF-to-trunk BF ratio was also found. WHR was also correlated with DXA derived BF distribution markers, but only in partial correlations controlled for age and sex.

Table 8.2 – Descriptive data of the studied sample study 5

	NAFLD Patients (n=28)			
Variables	**Mean ± sd ***		**Min. – Max.**	
Age, yr (median, yr)	49.5 ± 12.8	(49)	25 – 68	
Sex, n female (% female)	9	(32,1)		
Anthropometry				
Weight, kg (COV, %)	87.6 ± 12.7	(0,07)	66.2 – 115.8	
Height, cm (COV, %)	167.2 ± 9.2	(0,03)	149.5 – 183.7	
WC, cm (COV, %)	104.8 ± 10.6	(0,49)	85.3 – 128.7	
Hip-C, cm (COV, %)	107.6 ± 12.0	(0,38)	92.3 – 138.3	
Thigh-C, cm (COV, %)	52.9 ± 5.7	(0,47)	44.5 – 67.5	
Calf-C, cm (COV, %)	39.3 ± 3.4	(0,44)	32.9 – 46.4	
Arm-C, cm (COV, %)	34.0 ± 3.6	(0,64)	29.3 – 44.6	
Body Indexes				
BMI, kg/m^2 (% obese)	29.1 ± 4.0	(32.1)	22.6 – 42.2	
BAI	32.2 ± 8.2		21.0 – 51.0	
WHtR (% High WHtR)	0.63 ± 0.08	(100.0)	0.50 – 0.82	
WHR (% High WHR)	0.98 ± 0.07	(67.9)	0.85 – 1.11	
Whole and Regional Body Composition				
Whole BF, kg (%)	27.2 ± 9.3	(31.31 ± 8.20)	13.7 – 51.2	(18.84 – 46.28)
Whole FFM, kg (%)	58.7 ± 9.1	(68.69 ± 8.20)	39.6 – 77.7	(53.72 – 81.16)
Trunk BF, kg (%)	15.2 ± 5.2	(33.15 ± 7.65)	7.4 – 25.0	(20.87 – 48.01)
Trunk FFM kg (%)	29.9 ± 3.9	(66.85 ± 7.65)	21.1 – 38.6	(51.99 –79.13)
Append BF, kg (%)	10.8 ± 4.8	(30.42 ± 10.39)	5.2 – 25.7	(13.63 – 50.40)
Append FFM, kg (%)	24.5 ± 5.1	(69.58 ± 10.39)	14.9 – 34.8	(49.60 – 86.37)
Abd BF, kg (%)	3.5 ± 1.2	(37.57 ± 6.59)	1.7 – 6.3	(26.09 – 49.40)
CAbd BF, kg (%)	2.9 ± 0.8	(35.82 ± 5.70)	1.6 – 5.0	(24.28 – 44.64)
Body Fat Distribution (ratios)				
Trunk BF-to-Append BF ratio	1.477 ± 0.371		0.958 – 2.547	
Abdominal BF-to- whole BF ratio	0.130 ± 0.025		0.045 – 0.185	
Abdominal BF-to-Trunk BF ratio	1.231 ± 0.039		0.095 – 0.299	

COV – coefficient of variation; WC - waist circumference measured just above the iliac crest; Hip-C – hip circumference; Thigh-C – thigh circumference; Calf-C – calf circumference; Arm-C – arm circumference; BMI – body mass index; BAI – body adiposity index; Append – Appendicular; WHtR – weight-to-height ratio; WHR – weight.-to-hip ratio; BF – body fat; FFM – fat free mass; * Results are presented as mean ± standard deviation, unless otherwise noted; Min. – lowest observed value; Max. – highest observed value; BMI – body mass index; BF – body fat; FFM – fat free mass.

Table 8.3 – Partial and semipartial correlations between circumferences and body indexes and body fat content and distribution, controlled for age and sex.

Variables		Circumferences					Body Indexes			
		Arm-C	WC	Hip-C	Thigh-C	Calf-C	BMI	BAI	WHtR	WHR
Whole BF	r †	0.76*	0.79*	0.88*	0.74*	0.79*	0.65*	0.58*	0.63*	0.13
	r ‡	0.54*	0.55*	0.62*	0.53*	0.56*	0.45	0.41	0.45	0.09
Whole %BF	r †	0.45	0.56*	0.59*	0.40	0.49	0.40	0.54*	0.56*	0.15
	r ‡	0.24	0.30	0.31	0.21	0.26	0.21	0.29	0.30	0.08
Trunk BF	r †	0.64	0.87*	0.81*	0.64*	0.73*	0.52	0.56*	0.73*	0.35
	r ‡	0.51*	0.68*	0.64*	0.50*	0.58*	0.40	0.44	0.58*	0.27
Trunk %BF	r †	0.39	0.62*	0.54*	0.34	0.46	0.35	0.48	0.59*	0.29
	r ‡	0.26	0.41	0.36	0.23	0.30	0.23	0.32	0.39	0.19
Append BF	r †	0.78*	0.56*	0.84*	0.77*	0.74*	0.65*	0.52*	0.41	-0.16
	r ‡	0.52*	0.36	0.55*	0.50*	0.48	0.46	0.34	0.27	-0.11
Append %BF	r †	0.41	0.37	0.51*	0.35	0.42	0.35	0.52*	0.41	-0.06
	r ‡	0.18	0.17	0.23	0.16	0.19	0.17	0.24	0.19	-0.03
Abd BF	r †	0.09	0.75*	0.45	0.14	0.35	0.17	0.57*	0.82*	0.57*
	r ‡	0.08	0.67*	0.40	0.13	0.31	0.15	0.51*	0.73*	0.51*
Abd %BF	r †	-0.03	0.51*	0.27	-0.01	0.14	0.06	0.44	0.61*	0.44
	r ‡	-0.03	0.42	0.23	-0.01	0.12	0.05	0.37	0.51*	0.36
C Abd BF	r †	0.13	0.72*	0.43	0.26	0.36	0.11	0.48	0.74*	0.60*
	r ‡	0.13	0.70*	0.42	0.25	0.35	0.10	0.47	0.72*	0.58*
C Abd %BF	r †	0.06	0.51*	0.35	0.11	0.21	0.10	0.51*	0.61*	0.36
	r ‡	0.06	0.44	0.30	0.09	0.18	0.09	0.45	0.53*	0.32
Trunk BF-to-Append BF ratio	r †	-0.03	0.39	0.06	0.04	0.05	-0.08	-0.02	0.31	0.56*
	r ‡	-0.03	0.32	0.05	0.04	0.05	-0.06	-0.02	0.25	0.46
Abd BF-to- whole BF ratio	r †	-0.48	0.18	-0.21	-0.38	-0.21	-0.21	0.20	0.39	0.51*
	r ‡	-0.43	0.16	-0.19	-0.35	-0.19	-0.20	0.18	0.36	0.46
Abd BF-to-Trunk BF ratio	r †	-0.56*	-0.05	-0.33	-0.49	-0.33	-0.27	0.13	0.21	0.30
	r ‡	-0.54*	-0.05	-0.32	-0.48	-0.32	-0.26	0.12	0.20	0.29

Arm-C – arm circumference; WC – waist circumference ; Hip-C – hip circumference; Thigh-C – thigh circumference; Calf-C – calf circumference; BMI – body mass index; BAI – body adiposity index; WHtR – waist to height ratio; WHR – Waist-to-Hip ratio; Whole BF – whole body fat; Trunk BF – trunk body fat; Append BF – appendicular body fat; Abd BF – abdominal body fat; CAbd BF – central abdominal body fat; † - partial correlations between studied body circumferences or indexes and the studied BF content or distribution variables, controlled for age and sex; ‡ - semipartial correlations between studied body circumferences or indexes and the studied BF content or distribution variables, controlled for age and sex ; * - significant for $p<0.05$ and $\beta=0.20$.

Table 8.4 shows the results for partial and semipartial correlations between each independent and dependent variables, controlled for sex, age and BMI (except when BMI was an independent variable, in this case, control variables were age, sex and WC). When BMI was added has a control variable, all coefficients of correlation between clinical markers and whole BF were slightly reduced showing that BMI explained a some variation of the dependent variables already accounted for by the studied independent variables (the body circumferences and indexes). However BMI, together with sex and age, explained a more considerable amount of the variation of the dependent variables that could not be explained by the tested clinical markers, as can be confirmed

by the considerable drop in all coefficients of correlation obtained in semipartial correlations, as opposed to partial correlation, which resulted non-significant.

Table 8.4 – Partial and semipartial correlations between circumferences and body indexes and body fat content and distribution, controlled for age, sex, and body mass index.

Variables		Circumferences					Body Indexes			
		Arm-C	WC	Hip-C	Thigh-C	Calf-C	BMI	BAI	WHtR	WHR
Whole BF	r †	0.58*	0.68*	0.79*	0.58*	0.62*	0.43	0.35	0.48	0.19
	r ‡	0.31	0.37*	0.42	0.31	0.34	0.19	0.19	0.26	0.10
Whole %BF	r †	0.27	0.45	0.48	0.22	0.33	0.13	0,42	0,46	0,17
	r ‡	0.13	0.22	0.23	0.11	0.16	0.06	0.21	0.23	0.08
Trunk BF	r †	0.45	0.82*	0.71*	0.46	0.59*	0.19	0.37	0.64*	0.43
	r ‡	0.29	0.54*	0.47	0.30	0.39	0.07	0.24	0.43	0.28
Trunk %BF	r †	0.22	0.54*	0.44	0.17	0.31	0.03	0.36	0.52*	0.33
	r ‡	0.14	0.34	0.27	0.11	0.19	0.02	0.22	0.32	0.20
Append BF	r †	0.64*	0.32	0.70*	0.62*	0.53*	0.51*	0.26	0.15	-0.19
	r ‡	0.32	0.16	0.35	0.30	0.26	0.27	0.13	0.07	-0.09
Append %BF	r †	0.24	0.23	0.40	0.19	0.27	0.19	0.41	0.30	-0.06
	r ‡	0.10	0.10	0.17	0.08	0.11	0.08	0.18	0.13	-0.02
Abd BF	r †	-0.06	0.78*	0.45	0.03	0.31	-0.39	0.57*	0.84*	0.59*
	r ‡	-0.05	0.69*	0.29	0.03	0.27	-0.23	0.49	0.74*	0.52*
Abd %BF	r †	-0.11	0.57*	0.32	-0.06	0.14	-0.29	0.49	0.66*	0.45
	r ‡	-0.09	0.47	0.26	-0.05	0.11	-0.21	0.40	0.54*	0.37
C Abd BF	r †	0.04	0.77*	0.46	0.21	0.36	-0.41	0.48	0.77*	0.61*
	r ‡	0.04	0.74*	0.44	0.20	0.35	-0.28	0.46	0.74*	0.68*
C Abd %BF	r †	-0.02	0.53*	0.37	0.04	0.17	-0.21	0.53*	0.63*	0.37
	r ‡	-0.02	0.46	0.32	0.04	0.15	-0.16	0.47	0.55*	0.32
Trunk BF-to-Append BF ratio	r †	0.01	0.50*	0.13	0.10	0.13	-0.34	0.01	0.38	0.56*
	r ‡	0.01	0.41	0.11	0.08	0.11	-0.26	0.01	0.31	0.46
Abd BF-to- whole BF ratio	r †	-0.46	0.35	0.09	-0.33	-0.09	-0.37	0.38	0.57*	0.51*
	r ‡	-0.42	0.31	-0.08	-0.29	-0.08	-0.33	0.34	0.51*	0.46
Abd BF-to-Trunk BF ratio	r †	-0.53*	0.11	-0.21	-0.43	-0.22	-0.28	0.34	0.39	0.30
	r ‡	-0.50*	0.11	-0.20	-0.41	-0.20	-0.27	0.32	0.36	0.28

Arm-C – arm circumference; WC – waist circumference ; Hip-C – hip circumference; Thigh-C – thigh circumference; Calf-C – calf circumference; BMI – body mass index; BAI – body adiposity index; WHtR – waist to height ratio; WHR – Waist-to-Hip ratio; Whole BF – whole body fat; Trunk BF – trunk body fat; Append BF – appendicular body fat; Abd BF – abdominal body fat; CAbd BF – central abdominal body fat; † - partial correlations between studied body circumferences or indexes and the studied BF content or distribution variables, controlled for age, sex and BMI; ‡ - semipartial correlations between studied body circumferences or indexes and the studied BF content or distribution variables, controlled for age, sex and BMI; * - significant for $p<0.05$ and $\beta=0.20$.

The same occurred when BMI was the independent variable controlled for age, sex and WC. The same was also true for the prediction of trunk BF, except for WC which revealed to be consistently and strongly associated with trunk BF, and rather unaffected by controlling also for BMI. The relation between WC, WHtR and WHR with Abd BF and CAbd BF depots, did not seem to be influenced by the introduction of BMI as a control variable as well. Tough, again only WHtR was consistently and strongly associated with both absolute and relative values of both abd BF and CAbd. With the

introduction of BMI as a control variable WHtR was found to be correlated to Abd BF-to-whole BF ratio. The associations between WHR and arm-C with DXA derived BF distribution ratios remained fairly unchanged, when adding BMI as a control variable.

The coefficients of correlation obtained in partial and semipartial correlations between BMI and the studied DXA derived BF content and distribution variables were compared with similar coefficients of correlation obtained between all other independent variables (body circumferences and indexes) and the same studied dependent variables, as can be observed in tables 8.5 and 8.6. The correlation of WC and WHtR with Abd BF and CAbd BF was significantly superior, as compared to BMI, either when using only age and sex as controlling variables as when adding BMI to the control variables. WC is also consistently superior to BMI in the association with trunk BF. WHR seem particularly better surrogate of BF distribution than is BMI. WHtR was also found to be consistently superior to BMI in the prediction of Abd BF-to-whole BF, for a given BMI. BMI was found superior in the correlation with whole BF and appendicular BF, only when compared with WHR.

Table 8.5 – Z statistic P values for the comparison between the correlation coefficients obtained in partial and semipartial correlations, controlled for age and sex, between body mass index and all dependent variables, and between all studied body circumferences and indexes and the same dependent variables

Variables		Circumferences					Body Indexes		
BMI		Arm-C	WC	Hip-C	Thigh-C	Calf-C	BAI	WHtR	WHR
Whole BF	r †	0.414	0.308	0.030*	0.661	0.287	0.670	0.924	0.023*
	r ‡	0.677	0.619	0.384	0.724	0.605	0.862	0.989	0.165
Whole %BF	r †	0.816	0.444	0.367	0.997	0.669	0.516	0.453	0.247
	r ‡	0.911	0.731	0.690	0.991	0.840	0.766	0.737	0.640
Trunk BF	r †	0.521	0.008**	0.058	0.553	0.212	0.865	0.209	0.432
	r ‡	0.628	0.141	0.244	0.648	0.400	0.857	0.400	0.615
Trunk %BF	r †	0.870	0.213	0.406	0.965	0.663	0.590	0.264	0.822
	r ‡	0.898	0.470	0.612	1.000	0.773	0.731	0.513	0.909
Append BF	r †	0.253	0.629	0.113	0.384	0.521	0.498	0.240	0.001**
	r ‡	0.790	0.672	0.690	0.861	0.927	0.600	0.421	0.032*
Append %BF	r †	0.817	0.926	0.471	0.990	0.758	0.465	0.791	0.132
	r ‡	0.953	0.997	0.808	0.977	0.927	0.805	0.942	0.485
Abd BF	r †	0.757	0.005**	0.277	0.908	0.493	0.096	<0.000***	0.090
	r ‡	0.799	0.020*	0.336	0.934	0.536	0.150	0.006**	0.142
Abd %BF	r †	0.737	0.076	0.272	0.799	0.772	0.142	0.022*	0.145
	r ‡	0.785	0.159	0.527	0.837	0.809	0.238	0.074	0.241
C Abd BF	r †	0.934	0.004**	0.207	0.578	0.333	0.143	0.003**	0.040*
	r ‡	0.923	0.006**	0.215	0.576	0.339	0.152	0.004**	0.046*
C Abd %BF	r †	0.875	0.113	0.374	0.997	0.725	0.109	0.035*	0.345
	r ‡	0.901	0.172	0.436	0.992	0.751	0.168	0.076	0.406
Trunk BF-to-Append BF ratio	r †	0.882	0.085	0.645	0.674	0.648	0.845	0.167	0.013*
	r ‡	0.899	0.158	0.700	0.723	0.700	0.865	0.254	0.047*
Abd BF-to- whole BF ratio	r †	0.290	0.160	0.974	0.650	0.982	0.140	0.026*	0.006**
	r ‡	0.350	0.197	0.968	0.569	0.977	0.175	0.042*	0.013*
Abd BF-to-Trunk BF ratio	r †	0.207	0.436	0.789	0.347	0.795	0.157	0.090	0.041*
	r ‡	0.227	0.447	0.805	0.367	0.805	0.170	0.100	0.047*

Arm-C – arm circumference; WC – waist circumference ; Hip-C – hip circumference; Thigh-C – thigh circumference; Calf-C – calf circumference; BMI – body mass index; BAI – body adiposity index; WHtR – waist to height ratio; WHR – Waist-to-Hip ratio; Whole BF – whole body fat; Trunk BF – trunk body fat; Append BF – appendicular body fat; Abd BF – abdominal body fat; CAbd BF – central abdominal body fat; † - Comparison between coefficients of correlation obtained in partial correlations, controlled for age and sex; ‡ - Comparison between coefficients of correlation obtained in semipartial correlations, controlled for age and sex; * - significant for p<0.05; ** - significant for p<0.01; ***- significant for p<0.001.

Table 8.6 – Z statistic P values for the comparison between the correlation coefficients obtained in partial and semipartial correlations between all studied body circumferences and indexes and all dependent variables, controlled for age, sex and body mass index, and the coefficients obtained in partial and semipartial correlations between body mass index and the same dependent variables, controlled for age and sex.

Variables		Circumferences					Body Indexes		
BMI		Arm-C	WC	Hip-C	Thigh-C	Calf-C	BAI	WHtR	WHR
Whole BF	r †	0.696	0.840	0.295	0.692	0.887	0.156	0.392	0.042*
	r ‡	0.567	0.725	0.913	0.565	0.633	0.303	0.445	0.179
Whole %BF	r †	0.619	0.809	0.717	0.483	0.800	0.916	0.778	0.386
	r ‡	0.785	0.962	0.926	0.711	0.869	0.994	0.950	0.654
Trunk BF	r †	0.361	0.048*	0.280	0.751	0.743	0.492	0.517	0.670
	r ‡	0.679	0.522	0.757	0.378	0.731	0.545	0.905	0.651
Trunk %BF	r †	0.619	0.395	0.722	0.488	0.877	0.965	0.470	0.917
	r ‡	0.748	0.673	0.865	0.662	0.906	0.997	0.719	0.929
Append BF	r †	0.962	0.127	0.701	0.860	0.523	0.074	0.029*	0.001**
	r ‡	0.536	0.228	0.626	0.508	0.406	0.186	0.130	0.036*
Append %BF	r †	0.681	0.651	0.831	0.534	0.749	0.791	0.843	0.138
	r ‡	0.818	0.804	0.991	0.749	0.846	0.977	0.882	0.494
Abd BF	r †	0.413	0.002**	0.275	0.616	0.601	0.099	<0.001***	0.074
	r ‡	0.481	0.015*	0.595	0.669	0.646	0.167	0.005**	0.136
Abd %BF	r †	0.554	0.041*	0.345	0.661	0.794	0.095	0.009**	0.141
	r ‡	0.630	0.109	0.443	0.721	0.828	0.182	0.048*	0.238
C Abd BF	r †	0.806	0.001**	0.164	0.700	0.335	0.143	0.001**	0.035*
	r ‡	0.823	0.003**	0.180	0.701	0.349	0.158	0.003**	0.009**
C Abd %BF	r †	0.645	0.090	0.326	0.817	0.823	0.085	0.025*	0.332
	r ‡	0.694	0.151	0.395	0.851	0.838	0.145	0.064	0.400
Trunk BF-to-Append BF ratio	r †	0.774	0.028*	0.465	0.540	0.471	0.755	0.095	0.013*
	r ‡	0.807	0.075	0.542	0.608	0.547	0.791	0.171	0.048*
Abd BF-to-whole BF ratio	r †	0.316	0.039*	0.285	0.663	0.656	0.029*	0.002**	0.006**
	r ‡	0.394	0.063	0.660	0.718	0.675	0.050*	0.007**	0.014*
Abd BF-to-Trunk BF ratio	r †	0.258	0.175	0.845	0.500	0.851	0.028*	0.016*	0.040*
	r ‡	0.315	0.192	0.829	0.558	0.834	0.037*	0.023*	0.051

Arm-C – arm circumference; WC – waist circumference ; Hip-C – hip circumference; Thigh-C – thigh circumference; Calf-C – calf circumference; BMI – body mass index; BAI – body adiposity index; WHtR – waist to height ratio; WHR – Waist-to-Hip ratio; Whole BF – whole body fat; Trunk BF – trunk body fat; Append BF – appendicular body fat; Abd BF – abdominal body fat; CAbd BF – central abdominal body fat; † - Comparison between coefficients of correlation obtained in partial correlations, controlled for age, sex and BMI; ‡ - Comparison between coefficients of correlation obtained in semipartial correlations, controlled for age, sex and BMI; * - significant for p<0.05; ** - significant for p<0.01; ***- significant for p<0.001.

8.4. Discussion

To our knowledge the usefulness of some body circumferences and indexes in NAFLD patients, is unknown. The characteristics of the studied patients could be considered quite common for NAFLD patients, particularly the high levels of BMI, including obese and morbidly obese patients, because obesity have been identified as a strong risk factor for NAFLD and therefore highly prevalent in this sub-population (2-4, 6, 26). Some discrepancies were found in the diagnosis of adverse BC profiles, including excess BF, when using different methods and respective cutoff values. High WHtR was present in 100% of the patients meaning this may be more sensitive to identify patients with NAFLD even in those with normal BMI. All alternative methods identified higher prevalence of subjects considered to have a more adverse BC profile than did BMI. The high level found for WHtR, WHR and WC are consistent with the higher risk of comorbidities present in NAFLD patients, and therefore, though this was not the aim of the present study, our preliminary results suggest a preferable usage of the alternative methods in this population. Yet both sensitivity as well as specificity of these clinical markers and respective cutoffs should be thoroughly studied in NAFLD patients, in future studies.

About all of the studied body circumferences and indexes were strongly associated with whole BF but all seem to be particularly influenced by control variables age and sex, and particularly by BMI. BMI seems to slightly overlap the explanation of the dependent variables by the studied body circumferences and indexes, as can be interpreted from confronting coefficients of correlation obtained with and without using BMI as a control variable. Moreover BMI seems to considerably explain some variation of the dependent variables that could not be explained by the studied clinical markers, as can be inferred from the opposition of coefficients of correlation obtained in partial correlations to those obtained in semipartial correlations, both controlled for age, sex and BMI. Thus, when WC was included as controlling variable in the correlation between BMI and whole BF, the coefficient of correlation dropped quite significantly, particularly in semipartial correlation. This means WC explains very meaningfully the variation of BF and partially overlaps that explained by BMI. This was expected as all tested independent variables are known BC surrogates and, to some extent, may overlap and explained the same body component. However they have been shown to be related differently with different fat depots meaning that, though all being related to whole BF, they can explain partly different aspects of BF content (124). Appendicular and hip-C have been shown to be clearly more related to subcutaneous fat, while WC, WHtR and BMI have been found to be also

significantly related with visceral fat, in healthy adults (124, 208). BAI, advocated to be a superior marker of adiposity, particular related to whole %BF (24, 284, 285), did not stand out from the other markers, in the present sample of NAFLD patients.

Similar results to those found for the associations with whole BF were also found for the correlations with trunk BF, but in this case WC was always found consistently, independently and strongly associated with trunk BF, and exceptionally this association was found to be rather unaffected by controlling also for BMI. Therefore WC was considered to be the best marker of trunk BF between all tested. These results contrast with previous findings that showed, BMI and WHtR, in addition to WC, strongly related to trunk BF (263). However these previous findings, besides being collected from a sample of healthy adults, were not controlled for any covariates and therefore may be neither comparable to ours nor assumed to represent NAFLD sub-population specificity. WC was expected to be a good surrogate of trunk BF because it measures the circumference of the trunk, at the level of a specific abdominal landmark, and also because DXA assessed trunk BF has been shown to be highly related to visceral adipose tissue, assessed by MRI (91). Therefore our results are also in accordance to those observations showing WC to be highly related to visceral adipose tissue (124) and may increase the rational for the relation between WC and central obesity related metabolic abnormalities in NAFLD patients, as described for other populations (17, 23). The only consistent associations with appendicular BF were with Hip-C, Thigh-C and Arm-C. These results are in accordance to preferential relation of these body circumferences with subcutaneous adipose tissue rather than with visceral adipose tissue, which is only present in the trunk (124, 208). These results also support the inverse relation observed between these clinical markers and the risk of adverse outcomes (195, 213, 227). Yet, in the present study, these associations were not independent of BMI. Also in accordance to previous findings, in healthy adults (263), other markers in the present study, such as WC, BMI and WHtR, were also somewhat associated with appendicular BF, but the controlling variables age and sex seemed to be explaining a considerable part of the variation of the appendicular BF that was not explained by these clinical markers. This could be observed when the variation of appendicular BF accounted by the controlling variables was included in the correlation (semipartial correlation), resulting in coefficients of correlation that could not be found significant.

BF in the abdomen seems to be particularly associated to WC, WHtR and WHR, however only WHtR showed a strong association with all absolute and relative variables from both Abd BF and CAbd BF, unaffected by age, sex and BMI. Therefore WHtR was considered the best marker of abdominal fat accumulation in the present study. Both studied Abd BF and CAbd, as assessed by DXA,

were shown to be highly related to visceral adipose tissue, assessed by MRI (79, 80). Therefore our results suggest that WHtR may be an important tool to assess central obesity in clinical settings to help preventing and managing related metabolic risk, as have been described in other populations (17). This is consistent with the strong relation found between WHtR and the risk of hazardous metabolic outcomes, frequently associated with central obesity, including CVD and death (129, 254). Interesting is the fact that both sex and age unexpectedly did not explain much of the variation of the these central BF depots, confirmed by the rather small change observed between coefficients of correlation obtained from both partial and semipartial correlations, this may suggest a NAFLD subpopulation phenotype and warrants further investigation.

Concerning BF distribution, a rousing result is the consistent and independent inverse relation between arm-C and Abd BF-to-Trunk BF ratio, meaning that low arm-C may indicate more of trunk BF centered in the abdominal region. This is in accordance to the hazardous associations already found for low arm-C (226, 227). WHR was also correlated with DXA derived BF distribution markers, but only in partial correlations controlled for age and sex, meaning control variables explain a considerable amount of the variation of BF distribution that was not explained by WHR. These however contrasts with previous findings showing rather modest correlation coefficients in Pearson correlations between WHR and trunk BF-to-appendicular BF, consistently in both healthy men and women (263). In the present study the relation of WHR with BF distribution markers seems rather unaffected by BMI. Nonetheless, WHR was the only studied clinical marker to show any kind of relation with trunk BF-to-appendicular BF in the present study. We have showed that trunk BF-to-appendicular BF ratio was the best DXA assessed correlate with parasympathetic nervous system reactivation (data presented in study 1). Despite being a strong risk factor for metabolic and cardiovascular hazardous outcomes (18, 298, 299, 305-309, 312), the mentioned autonomic nervous system marker is linked to adipocyte functioning (326, 335, 342) and may have a role in BC abnormalities, either as a cause or as a consequence, however this needs further supporting basic and clinical evidences. Surprisingly, the tested clinical markers were not acceptable independent predictors of trunk BF-to-appendicular BF.

The comparison of coefficients of correlation obtained in partial and semipartial correlations between BMI and the studied DXA derived BF content and distribution variables with similar coefficients of correlation obtained between all other independent variables (circumferences and indexes) and the same studied dependent variables, as observed in tables 8.5 and 8.6, confirmed WC and WHtR as the best alternatives to BMI in the clinical assessment of BC, considering they are not

inferior to BMI in assessing whole BF but seem far superior in assessing central BF content and distribution. Not surprisingly WHR seems particularly superior to BMI in assessing BF distribution. WHR was the only tested clinical marker associated with trunk BF-to-appendicular BF which we have showed to be the best predictor of cardiac autonomic control variation and therefore more related with autonomic nervous system functioning (data from study 1), thus WHR has been assumed as beneficial for the diagnosis of NAFLD (252) reinforcing the beneficial use of WHR in clinical setting to improve patients BF distribution profile assessment, which is known to be associated with health risks, beyond overall adiposity .

The present study confirms the usefulness of BMI as a surrogate of whole BF however it emphasizes also its limitations in the assessment of both central BF accumulation and BF distribution, and also underscores that BMI neither superior to other tested BF surrogates, nor independent of WC in assessing nearly all BF depots in NAFLD patients. The present study also supports the usage of both WC and WHtR, as good alternative methods to BMI, to assess BC profile of NAFLD patients in clinical settings. Both arm-C and WHR may add information concerning BF distribution. The use of a simple measuring tape can be an inexpensive, simple and preferable way of assessing sound BC information in routine clinical appraisals in NAFLD patients, even when compared to commonly used BMI.

Chapter 9 – Conclusions

"Overall conclusions and recommendations to be drawn from this thesis"

In this chapter all five studies are linked as one single research and the overall final conclusions, supported by the finding of the present thesis, are presented. The chapter is divided in three sections: the first section of final conclusions regards what were considered to be the main strengths and limitations of the present thesis and the comprised studies; a second section of the present chapter highlights the main findings; ultimately we will point some final recommendations and future directions of research.

9.1. Strengths and limitations

There are several strengths and limitations to this thesis. We consider the link between science and practice sought by the present thesis to be a major strength of this work. This is intended to be supportive of clinical practice and, to that extent, we think that intention may have been achieved, with strong evidence and scientific support. The used body composition (BC) assessment reference method (DXA) albeit considered a gold standard instrument to assess BC in a three compartment model (24), is unable to determine visceral adiposity independently from subcutaneous body fat (BF). Nevertheless, recent studies indicate strong correlation between abdominal BF estimated from selected ROI, assessed by DXA, and visceral BF quantified by magnetic resonance imaging (79) and computed tomography (90, 374). The study design, cross-sectional, does not permit to establish the usefulness of the studied clinical variables in monitoring BF content and distribution changes over time, or to establish causal relationships between studied variables, based on the present results. These results represent, however, a preliminary analysis to establish the usefulness of some clinical BC surrogates, particularly body circumferences and indexes, as surrogates of BF content and distribution in non-alcoholic fatty liver disease (NAFLD) patients. Also, the size of the sample did not allow for the identification of significant associations lower than r=0.50, in order to attain minimal statistical power of 80% and statistical significance of 5%. Even though results seem fairly unexposed to type 1 and type 2 errors, the ability of detecting lower, still important, significant associations between variables was limited. However the aim of the present study does not seem to have been overlooked, because it sought to find the best clinical markers, which are found at the higher end of correlational range. Also, the recruitment strategy used to constitute the studied sample limits the external validity of our results, therefore, even though these results may constitute important preliminary findings in the population of NAFLD patients, they can only be applied to patients with the same characteristics to those included in the studied sample.

9.2. Main Findings

The main overall conclusions to be drawn from the present work can be divided into three sets. The first set of conclusions regards the first listed purpose of the present thesis: to determine if, and to what extent, specific markers of BC and BF distribution, are related with a selected mortality and cardiovascular risk related autonomic nervous system (ANS) marker in NAFLD patients. We could observe in the present thesis that increased BF content, particularly central BF, was associated with slow heart rate recovery (HRR) in NAFLD patients. BF distribution appears to be more important than overall BF accumulation in explaining the variation of HRR. Considering these observations we could conclude that the accumulation of central BF is somewhat linked to ANS decline though the cause/effect direction remains to be clarified. There appears to be also a particular BF distribution phenotype, regardless the amount of BF, that is related as well to ANS decay, hence again it remains to be disclosed if it is a specific centrally distributed BF phenotype that leads to altered ANS functioning, or if it is the other way around. In any case, both central BF accumulation and centrally distributed BF can both be related to higher cardiovascular risk in NAFLD patients, resulting from their inverse association with HRR, which, when under specific cutoffs, is a known strong risk factor for metabolic and cardiovascular abnormalities as well as cardiovascular and all-cause mortality.

A second set of conclusions to drawn from the present thesis is related to listed purposes 2 to 4: (purpose 2) to find which of the most used waist circumference measurement protocol (WCmp) is preferable to be used in clinical practice with NAFLD patients; (purpose 3) to analyze whether the most used WCmp affect the strength of association between waist-to-height ratio and both, whole and central BF in NAFLD patients; (purpose 4) to analyze whether the most used WCmp affect the strength of association between waist-to-hip ratio (WHR) and BF content and distribution in NAFLD patients. All studied waist circumferences performed similarly well in relating with analyzed BF depots, particular with more central accumulated BF, which endows waist circumference (WC) as an important risk assessment tool in NAFLD patients, considering central BF is known to be particular important in developing NAFLD and other metabolic and cardiovascular diseases, and was shown to be related with slow HRR as mentioned in the previous set of conclusions. The WC that was found most suitable for generalization in routine clinical appraisals was that measured just above the iliac crest. This protocol was quite similar to the others tested when analyzing the relations with whole and central BF content but it demonstrated three main advantages: it uses a bony landmark which has been considered an important feature for standardization, therefore is preferable to the WC

measured at the minimal torso, also called minimal waist, as well as to the WC measured at the umbilicus; it uses only one landmark, which has been sensed to be more practical and easy to identify and measure, in opposition to the WC measured at the mid distance between lowest rib and iliac crest, which has two landmarks and demands the calculation and identification of a third landmark; and finally the measurement is less time consuming in comparison to the other tested WC that used bony landmarks. WC measured just above the iliac crest also performed well when used to calculate both WHtR and WHR (purposes 3 and 4). It allowed strong associations between those body indexes, particularly with central BF and was found to be among the preferable solutions for both mentioned indexes. So, base only on these practical criteria we conclude that this should be the preferable WC to be included in routine clinical practice. Both WHtR and WHR performed well in the association with BF, particularly with central BF. As WC alone, also both WHtR and WHR seem good markers of metabolic and cardiovascular risk, and may be related to increased cardiovascular and all-cause mortality, given the inverse association of central BF with slow HRR. We conclude also that the WC measured just above the iliac crest should be the WCmp used to calculate both WHtR and WHR, considering the following four reasons: when using WC alone, WC measured just above the iliac crest was considered to be the preferred protocol; this preferred WCmp performed well when used to calculate both WHtR and WHR; WC measured just above the iliac crest was always found among the preferred solutions for the calculation of both mentioned body indexes and was never considered inferior to the other tested protocols in any criteria; and also standardization is an important purpose when the goal is to accomplish generalization, so it would be a strong barrier to generalization if one used different WCmp for each body index.

The third set of conclusions drawn from the present thesis regards the listed purposes 5 and 6: (purpose 5) to analyze how body circumferences and indexes perform as surrogate of whole and regional BF content and BF distribution in NAFLD patients; and (purpose 6) to find if any specific body index and/or circumference perform better than the commonly used body mass index (BMI) as surrogate of BC in NAFLD patients. The present thesis confirms the usefulness of BMI as a surrogate of whole BF however it emphasizes also its known limitations in the assessment of both central BF accumulation and BF distribution, and also underscores that BMI is neither superior to other tested BF surrogates, nor independent of WC in assessing nearly all BF depots in NAFLD patients. Among all tested body circumferences only WC was found to be a useful marker of BC, particularly of Central BF, in NAFLD patients. WHtR was the body index most related with BC, particularly with central BF and BF distribution, in the studied NAFLD patients. These were already reported in the present

thesis, except for BF distribution, and reinforce the usefulness of both WC and WHtR as risk assessment tools in NAFLD patients. Based on the results reported in the present thesis we are to conclude that both WC and WHtR are preferred alternative methods to BMI, to assess BC profile of NAFLD patients in clinical settings. Both arm circumference (Arm-C) and WHR may add information concerning BF distribution however this needs confirmation.

9.3. Recommendations for future research

Future studies should account for the limitations found in the present thesis, particularly that related with the sample size, in order to increase statistical power and to be able to detect meaningful results of smaller dimension. An increased sample would allow also testing differences between subgroups (e.g. men *vs* women; fit *vs* unfit; obese *vs* overweight *vs* normal weight; High HRR *vs* Low HRR, and other). The chosen marker of ANS functioning is known to be indicator of parasympathetic reactivation after maximal exercise, yet, in future studies it would be interesting to assess also the relation with both sympathetic and parasympathetic functioning using other methods. In the results from the present thesis both sex and age unexpectedly did not affect much of the associations with the central BF depots, and this was mentioned to possibly suggest a NAFLD subpopulation phenotype, though this need to be addressed in future research also. The continuation of this research should also focus on further associations with two main groups of variables: one group intends to deepen the present research and consists of assessing BC with different reference methods (e.g. magnetic resonance spectroscopy) to study other BF depots, including the liver itself, and BF distribution variables to test which are the best clinical surrogates to use in clinical settings with NAFLD; another group aims to broaden the present research and consists of testing other associations with different risk factors to find the best clinical marker of BC to use in clinical settings with NAFLD. Finally, a longitudinal approach may help clear some questions rose by the reported results, particularly the cause/effect relation of the reported association of central BF and BF distribution with ANS function, in other words, which abnormalities occur first and which follows. A longitudinal approach could also help to establish the best clinical marker to monitor changes in BC and associated risk profiles. Most important would be to test interventions in the population of NAFLD patients, particularly that focusing on lifestyle approaches, including increased physical activity, looking at specific health benefits related to BC changes as well as the benefits that are independent of BC changes resulting from increased physical activity or other features of the interventions to be studied.

Chapter 9 – Conclusions

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